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CONTENTS: TABLE DES MATIÈRES: INHALT:

F. C. Gerretsen, Studies in photosynthesis 1

V. L. Sheldon, Wm. G. Blue and *Wm. A. Albrecht,* Biosynthesis of amino acids according to soil fertility 33

Wang T. L., Nouvelle méthode de dosage des éléments minéraux fertilisants du sol par les azotobacter 41

B. G. Chitwood, Root-knot nematodes 47

P. R. Stout, W. R. Meagher, G. A. Pearson and *C. M. Johnson,* Molybdenum nutrition of crop plants 51

Jane Meiklejohn, The effects of glucose on impure cultures of nitrifying bacteria 88

H. W. Johnston, Amendment to Gerretsen's technique for the preparation of plates used for studying phosphate-dissolving micro-organisms 94

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STUDIES IN PHOTOSYNTHESIS

IV. REDOXPOTENTIALS AND CORRESPONDING pH CHANGES IN ILLUMINATED CRUDE CHLOROPLAST SUSPENSIONS

by F. C. GERRETSEN

Agricultural Experiment Station and Institute for Soil Research T.N.O. Groningen.

Introduction. From the preceding paper [12]) it is evident that chloroplast suspensions consume oxygen in the dark as well as during illumination and generally in the latter case at a much higher rate than in the former.

As organic material is oxidized, carbon dioxide will be produced and consequently the reaction will become more acid. As a matter of fact redoxpotential changes were often accompanied by characteristic pH changes; although these changes were relatively small they were very distinct and regular. Accurate pH determinations proved to be of great value in the interpretation of the phenomena induced by illumination of these crude chloroplast suspensions. This is especially true with regard to the cause of the so called induction period and to the possibility of carbon dioxide taking part in the oxidation reduction reactions which take place in these suspensions under anaerobic conditions.

Experimental methods. Redoxpotentials and pH's (glass electrode) were determined with the apparatus already described in the second paper; the accuracy being 0.1 mV, which corresponds to approximately 0.0017 pH units.

Readings were generally made every 5 minutes, but where rapid changes in reaction were occurring, readings were made every two or three minutes. In those cases where the potential or the reaction changed very slowly, observations were made less frequently.

In certain cases, in order to be able to calculate the amounts of carbon dioxide produced or consumed during irradiation, the buffering capacity of the suspension was determined by titrating the suspension with a $^1/_{100}$ norm. solution of KOH or HCl at the end of the experiments. For this purpose a microburette was connected

by means of a small rubber tube to one of the inlet tubes of the electrode vessel; care was taken to fill this tube first before any readings were made.

Results.

Experimental material used. 500 mg leaf from healthy oatplant grown out of doors (March-June) were suspended in 20 ml buffer solution and crushed, filtered and centrifuged. Temp. 24°C; initial pH 6.72. Air above the suspension.

The general course of an experiment under aerobic conditions, in which redoxpotentials and pH's were observed simultaneously is graphically represented in fig. 1. The redoxpotential changes are of the same nature as those described in the previous paper; in this case there is a distinct induction period, *BC*, during which there is a decrease of the potential which is subsequently followed by a rapid increase. It takes 7 minutes before the Eh has reached again the value it had at the end of the dark period.

When at *D* the light is turned off, the potential decreases rapidly in the dark; during the second illumination a potential of 356 mV is reached.

The pH curve shows that during the initial dark period the suspension becomes more acid (OP), due to the production of CO_2 by the residual respiration. As soon as illumination is started the suspension becomes more alkaline (PQ), the increase in alkalinity goes on for $\pm$ 9 minutes and coincides with the induction period observed in the redoxpotential. After that the reaction becomes more acid and the decrease is much quicker than in the initial dark period.

The decrease of the pH in the second and third dark periods is not so rapid as that during illumination, which means that illumination has a decided influence on the acidification of the suspension. This was to be expected, as during photo oxidation more CO_2 will be produced than in the dark.

When the rates at which the pH decreases in the successive dark and illumination periods are compared the mean rate in the first dark period being reckoned as 100, we find the following ratio's:

First dark period	100
First illumination period	300
Second dark period	160
Second illumination period	190
Third dark period	130

It is evident that illumination not only increases acidification but also has a distinct residual effect, which causes acidification to proceed in the subsequent dark periods at a rate 30–60% higher than in the initial dark period.

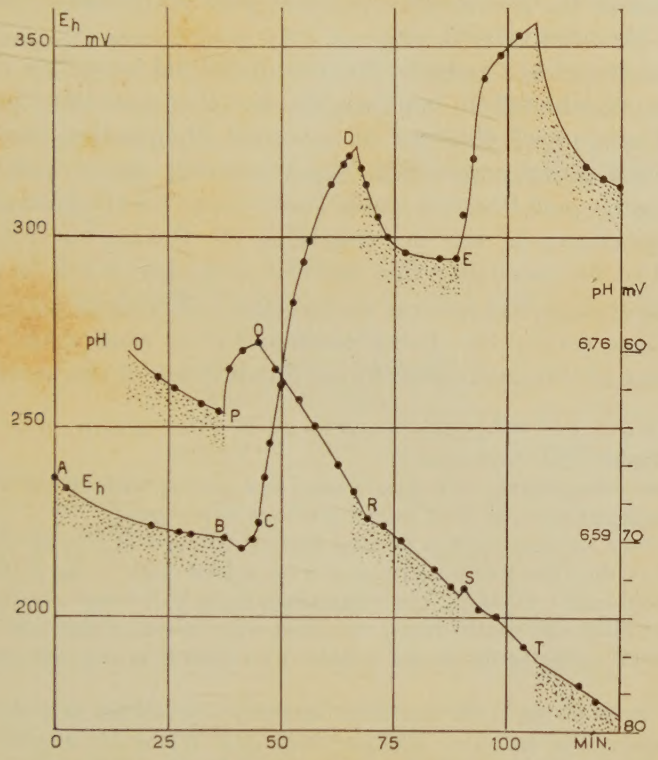

Fig. 1. General course of the changes in redox potential and pH of a crude chloroplast suspension under aerobic conditions. In the dark periods the suspension becomes more acid due to CO_2 production during residual respiration. On illumination a short, distinct, induction period is observed, during which the suspension becomes more alkaline PQ and the Eh decreases BC. This is not observed on a second illumination. (Black dots indicate that the suspension is in darkness).

In the preceding paper it was shown that in a number of cases the potential continued to increase after the light had been turned off; in the present case the increased acidification in the dark after a period of illumination might be traced to the same cause, i.e. the persistance of some intermediate oxidative compound formed during the photo oxidation of water.

A distinct decrease in acidity from the commencement of the *first* illumination period has been observed several times, though it is not always so pronounced as in this case. This might be caused either by the production of some alkaline substance, by the removal of H ions, or by the consumption of an acid compound, eventually by the liberation of OH ions.

It was tempting to ascribe the rise of the pH to carbon dioxide fixation or assimilation, however, the fact that a similar change in the pH was never observed at a second illumination made this assumption rather improbable. The irreversible photo oxidation of some organic acid however would easily count for the behaviour of the suspension; as the investigations of Bukatsch [4]) had pointed to the possibility that ascorbic acid plays a role in photosynthesis it seemed of interest to investigate whether or not ascorbic acid was present in the *Avena* leaves and if so, what would be the behaviour of this compound during illumination of the suspension.

Method for the determination of ascorbic acid in the crushed leaf suspension [8]).

2 g leaf were ground with quartz sand in a mortar with 30 ml H_2O and 30 ml buffersolution as used in the previous experiments.

The crude suspension was filtered and centrifuged at low speed.

10 ml of the clear green suspension were mixed with 10 ml meta-phosphoric acid and 1 ml H_2O. The meta-phosphoric acid coagulates the proteins and other cell constituents, including chlorophyll, which then can be removed by centrifuging, giving a clear supernatant having a faint yellow colour.

5 or 10 ml of the fresh indicator solution were added to 5 ml of the centrifugate. The indicator solution contained 25 mg 2–6 dichloro indophenol dissolved in 200 ml H_2O, a few drops of $^1/_{10}$ NaOH being also added. Care had to be taken that the indicator was not fully decolourised by the suspension; when this occurred, more indicator had to be added. The mixture was finally titrated untill colourless with a standard solution of ascorbic acid. This solution consisted of 10 mg ascorbic acid in 100 ml H_2O and 100 ml 4% HPO_3. The titre of the indicator solution was determined in the same way.

The determinations were made in duplicate and the mean error amounted to $\pm$ 2 μg ascorbic acid.

Experimental material used. 2 g of leaf from a healthy oatplant, grown outdoors between May and July, were suspended in 30 ml H_2O, and 30 ml. saltsolution without $NaHCO_3$. After filtering and centrifuging, 15 ml of the suspension were placed in a flat Warburg flask. Air above the suspension. Temp. 23°C. Illuminated with carbon arc light.

In the experiments I and II (table I), the original suspensions without any addition were used. In the IIId and IVth experiments 1,5 and 1 mg ascorbic acid dissolved in 1,5 ml water were added respectively. After the redoxpotential and pH changes in the dark had been observed for some time, the suspension was illuminated. As a control a second flask with the same contents was kept in the dark at the same temperature. At the end of the experiment the ascorbic acid content of the suspensions in both flasks was determined and compared with that at the beginning of the experiment. The results of these experiments are summarized in the following table.

TABLE I

The influence of illumination on the ascorbic acid content of crushed *Avena* leaf suspensions
(μg asc. acid in 15 ml suspension)

	at beginning of experiment	remaining after illum.	remaining in dark	mean loss per minute	
				dark	light
I. Original suspension no asc. acid added	493	63 (27′)	160 (104′)*)	3.2	6.8
II. Same as I, another plant (see fig. 2)	510	0 (16′)	217 (101′)	2.9	17.6
III. 1.5 mg asc. acid added	2351	133 (36′)	1660 (120′)	5.8	42.7
IV. 1.0 mg asc. acid added (see fig. 3)	1497	0 (55′)	309 (102′)	11.6	17.2

*) The total duration of the experiment is noted in brackets, and the duration of the illumination is given in brackets in the other column.

In calculating the loss of ascorbic acid during illumination, one has to bear in mind that f.i. in experiment I at the moment the light was turned on, the experiment had run 104 — 27 = 97 min. The amount of asc. acid was, at that moment 27 × 3.2 μg larger than at the end of the experiment in the dark i.e. it was 160 + 86 = 246 μg.

The loss during illumination has been 246 — 63 = 183 μg, or 6.8 μg per minute.

It is evident that the mature leaves of *Avena* plants have an ascorbic acid content varying between 1.0 to 1.7 mg per gram wet material. Clark [5]) found 0.4–0.7 mg ascorbic acid in the tips of Avena coleoptiles and Balk and Pommer [3]) 0.7 mg in oat-plants 16 days old, both calculated on a wet weight basis. Furthermore the experiments show that whilst ascorbic acid disappears in the dark the rate of disappearance during illumination of the suspension is much quicker. According to Hamner the loss of vitamin C in

crushed cells is very rapid and especially in cases where the enzymes retain their activity and the suspension is exposed to oxygen or the products of the photo-oxidation of water.

From the fact that often the acidity increases in the dark (fig. 1 *OP*) and even more quickly during illumination (fig. 1 *QR*) it may be concluded that the ascorbic acid is not only oxidized to dehydro-ascorbic acid, in which case the pH would remain constant, but that it undergoes considerable further decomposition. This explains why at a second irradiation there is no, or only a faint, indication of a sudden decrease in acidity such as is to be observed when the fresh suspension is illuminated for the first time.

From table I it can be seen that the rates at which ascorbic acid is oxidized differ widely. The cause of this phenomenon has not been traced. It must however be remarked that in experiments II and IV there is a possibility that the rate of oxidation during illumination has been higher than that noted in the table, namely when the stage of complete disappearance of the ascorbic acid was reached earlier in the experiments.

The results of the experiments II and IV are graphically represented in figs. 2 and 3. Fig. 2 represents the case of a healthy outdoor grown oatleaf, containing 1,05 mg ascorbic acid per gram wet material. In the dark period the reaction slowly becomes more alkaline as the ascorbic acid is oxidized; immediately the suspension is illuminated the reaction becomes more alkaline much more rapidly until at *C* the suspension commences to become acid, rapidly at first and more slowly later. At *C* the suspension was aerated by shaking and to some extend this will have caused the sharp increase in acidity and the corresponding increase in the Eh. However, the shape of the second curve (fig. 3) shows the same three different phases. After the sudden change in reaction resulting from acidification by the 1 mg ascorbic acid added to the suspension, the reaction becomes more alkaline, very slowly at first, due to oxidation of the ascorbic acid in the dark (fig. 3, *AB*). The change in the reaction towards alkalinity on illumination is very sudden and steep, and in this case also there is at *C* an initial very rapid increase in acidity followed by a slower increase later. In the experiments represented in fig. 3 the suspension was shaken throughout the experiment.

The change in the reaction is the resultant of the disappearance of the ascorbic acid and of the production of CO_2 as the final oxida-

tion product of the ascorbic acid and of the cell constituents involved in these processes. From the fact that the pH curve shows

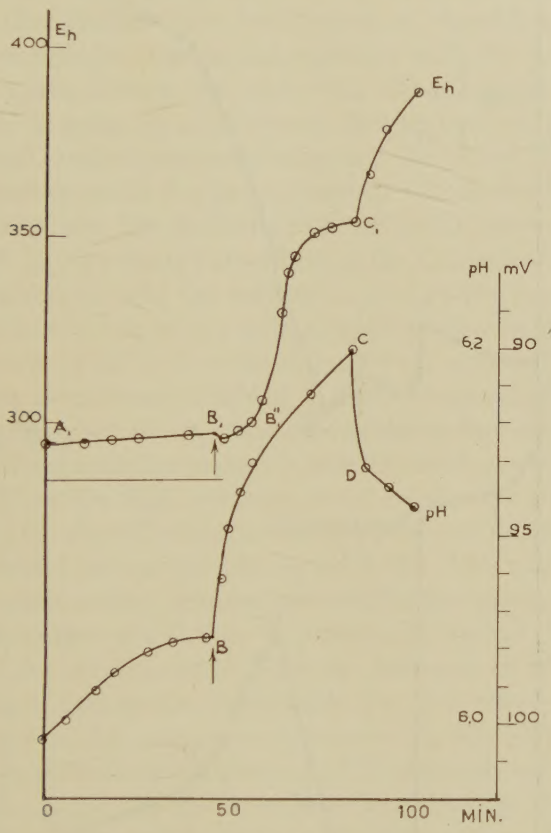

Fig. 2. An exceptionally prolongued induction period in a suspension which originally contained 1,05 mg ascorbic acid per gr leaf (wet). At C air was shaken into the suspension. The acidity decreases because the ascorbic acid is destroyed and increases by reason of the CO_2 developed during its final destruction. (↑ indicates that the light is turned on)

such a distinct turning point it may be concluded that the dehydrogenation of the ascorbic acid occurs in two steps: there is a loss in acidic groups, which is immediately followed by an extensive decomposition of the residues of the molecules which is accompanied by marked CO_2 production, resulting in an increase in acidity. At *D* (in both figs. 2 and 3) destruction seams to be complete, the slow

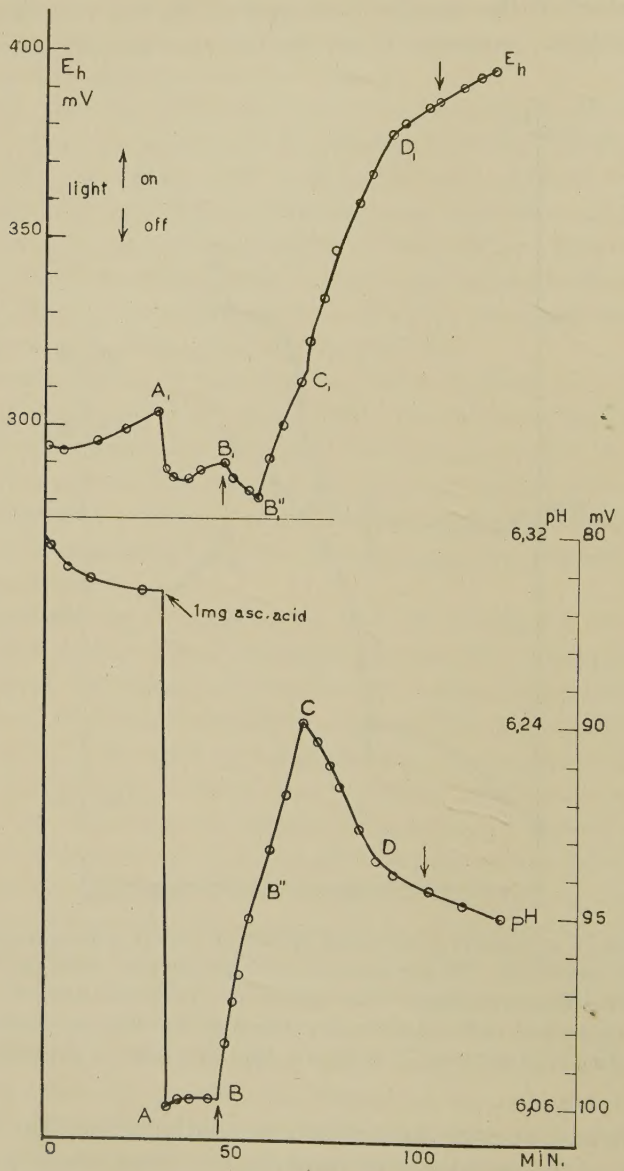

Fig. 3. In this case 1 mg ascorbic acid had been added to the suspension, which was continually shaken with air. Note the resemblance with the pH curve of fig. 2.

acidification at this stage being due to CO_2 produced during the oxidation of the cell constituents.

From the Eh curve in fig. 2 it is evident that before an actual rise sets in after the light has been turned on, there is a lag period B_1–B_1'' of 5–6 minutes; this period coincides with the steepest part of the pH curve during the time that dehydrogenation of the ascorbic acid is going on vigourously. In this case we are dealing with a normal crushed leaf suspension and the period B_1–B_1'' is the induction period which has been observed repeatedly throughout these investigations. The declining slope of the Eh curve just before C is caused by increasing exhaustion of the O_2 content of the suspension; shaking air into the suspension at C causes an immediate rise of the Eh, which coincides with a rapid increase in acidity.

The Eh curve of fig. 3 shows a drop of 18 mV occurring immediately after the ascorbic acid is added. It is to be expected that the Eh would increase when the medium was acidified but since ascorbic acid has a strong reducing capacity, which is more powerful, the Eh decreases. When the light is turned on at B_1 there is a sudden decrease in the Eh (B_1–B_1'') which lasts 8 minutes and coincides exactly with the steepest part of the pH curve (B–B''). This may be due to the normal relationship between pH and Eh (an increase in alkalinity causing a decrease in Eh) or to an intensification of the reducing capacity of the ascorbic acid under the influence of illumination. Probably both factors are responsible for this effect for at the moment when the Eh commences to increase rapidly (B_1'') alkalinisation of the suspension is still going on (B''), although at a somewhat reduced rate. If the change in reaction was the only cause of the decrease in Eh, this would certainly continue until C. The sudden change to an increase in Eh at B_1'' most probably indicates saturation of the double bond which is responsible for the powerfull reducing capacity of ascorbic acid.

At C where an increase in alkalinity suddenly changes to an increase in acidity, the Eh curve shows a steep slope (C_1D_1) as may be expected and when the rate of increase of acidity begins to slow down at D the Eh correspondingly shows a slower rate of increase.

The most important result of these experiments is that it has proved possible, by adding small amounts of ascorbic acid, to prolong the induction period observed when the leaf suspension is exposed to light for the first time. The similarity between the pH

and Eh curves of a normal suspension and those of a suspension to which ascorbic acid had been added make it highly probable that this first induction period has to be ascribed to the presence of ascorbic acid in the leaf suspension.

Changes in the pH and redoxpotential of crude chloroplast suspensions under anaerobic conditions. As it is to be expected that the reducing properties of the chloroplast suspensions would be more pronounced in the absence of oxygen it seemed of interest to observe the changes in pH and redoxpotential under anaerobic conditions.

Experimental material used. 1,5 g of leaves from oat-plant, grown in the greenhouse under conditions of artificial illumination for $2^1/_2$ months (Sept.–Nov.); suspended in 45 ml 0,005 N sodiumcarbonate buffersolution. Temp. 21°C. Pt electrode, initial pH 7,05.

At the beginning air above the suspension, after 83 min. nitrogen bubbled through the suspension (fig. 4).

During the first dark period there is a steady increase in the acidity of the suspension due to CO_2 production by the residual respiration. (Decrease of the pH until illumination at A.)

At the same time the Eh increases, indicating that substances responsible for the redoxpotential are passing to a higher stage of oxidation. As soon as the suspension is illuminated, the reaction suddenly becomes more alkaline, until after 6–7 minutes this is followed by an increasing acidity as has been discussed in the preceding chapter.

The Eh (upper curve) shows a sudden rise of very short duration (3 min.) followed by a rapid decrease; this indicates that the oxygen content of the suspension was nearly exhausted when illumination commenced and that photo oxidation has changed to photoreduction. This means virtually that hydrogen transfer during dehydrogenation is so accelerated by illumination, that the amount of oxygen diffusing into the suspension is inadequate to accept all the activated hydrogen; consequently other substances, some of which determine the redoxpotential of the suspension, act as hydrogen acceptors, which results in a lowering of the redoxpotential.

When at *B* the light is turned off, acidification proceeds at a higher rate than during illumination. Though the cause of this phenomenon is not quite clear it stops as soon as the air above the suspension has been replaced by nitrogen. As the pH remains prac-

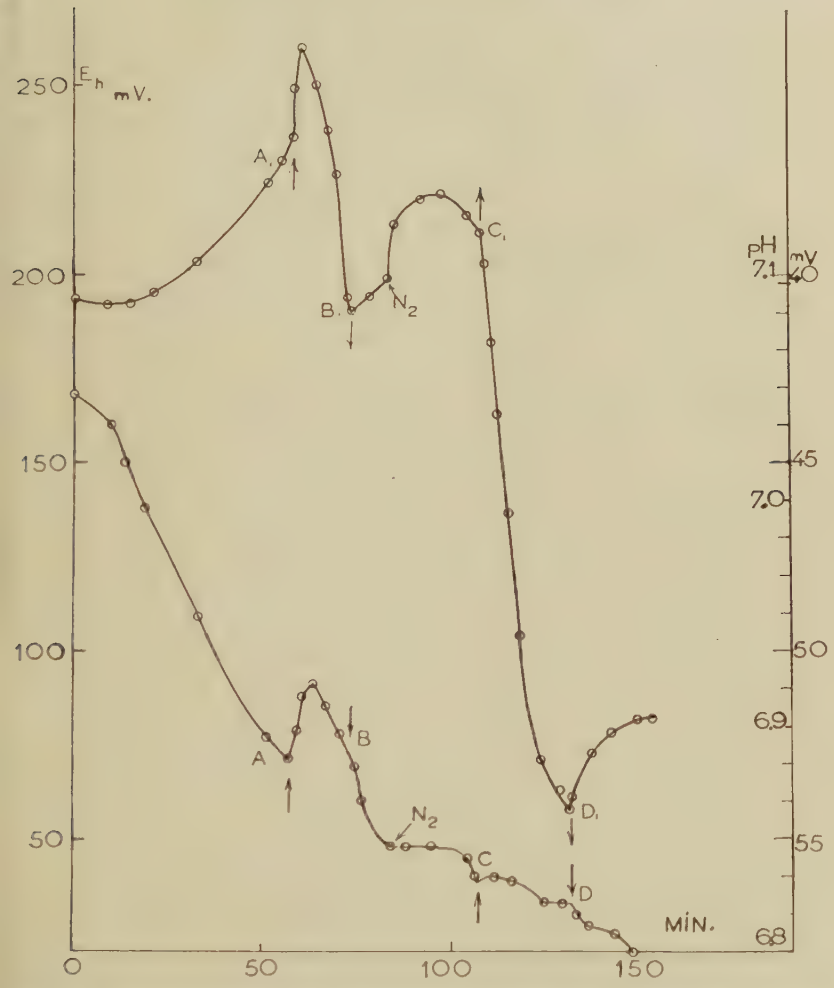

Fig. 4. This experiment was commenced under aerobic conditions, with air above the suspension. In the first dark period the pH (lower curve) decreased due to CO_2 production by residual respiration. On illumination (↑) the pH showed a distinct induction period AB, which was absent in the second illumination period at C. The Eh of the suspension (upper curve) increased on illumination at A_1, but the course of the curve was reversed after a few minutes, when the oxygen in the suspension was exhausted. After nitrogen has been introduced the Eh rapidly decreases on illumination, followed by a sharp increase when the light is turned off. (↓)

tically constant during the second illumination period it is obvious that the acidification in the previous periods is due largely to CO_2 produced by oxidation of the organic cell constituents by the residual oxygen in the suspension.

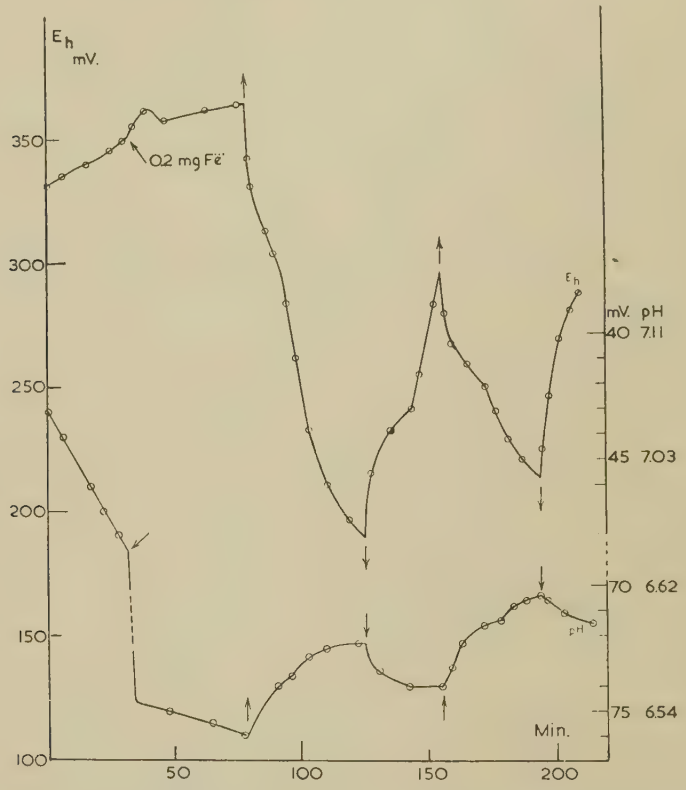

Fig. 5. To this suspension 0,2 mg Fe^{+++} as ferric ammonium sulphate had been added, which intensified the reducing capacity. The interesting point is that here the suspension becomes more alkaline during illumination, whereas in the dark periods acidification prevails.

During replacement of the air above the suspension by nitrogen the suspension was unavoidably agitated and thereby came in contact with the residual oxygen containing atmosphere above the suspension, consequently the Eh increases temporarily but within 10 minutes a decrease sets in, which is greatly accentuated when the light is switched on (C_1D_1). The decrease in the Eh amounts to 150

mV in about 15 minutes and the potential reaches a value of 59 mV (referred to normal hydrogen electrode) which is approximately the potential of a 50% reduced methylene blue solution at pH 5.7. When the light is turned off at *D*, the potential increases by 25 mV, the reaction at the same time becoming more acid. This is regularly observed in an anaerobic medium when the Eh has reached a low value after a period of illumination.

The pH curve of the experiment described on pages 177 and 178 of the second paper of this series (0.2 mg ferric amm. sulf. added to 9 ml suspension) showed typical changes and is reproduced in fig. 5, notwithstanding the fact that the experiment was not started under anaerobic conditions. In the beginning there is a rapid decrease of the pH due to residual respiration; the acid reaction of the ferric ammonium sulfate caused the pH to drop from 6.98 to 6.54. When the suspension is illuminated the pH increases, the suspension becoming more alkaline, whereas when illumination ceases the course of the pH changes is reversed. During the second period of illumination and the second dark period the same changes are repeated. The Eh changes are very pronounced and in the opposite direction to the pH changes. Although the suspension was not kept under anaerobic conditions this behaviour is typical for a suspension devoid of oxygen.

The question may be raised as to whether these typical pH changes have to be ascribed to the presence of the ferric ammonium sulfate or to some other cause. The reduction of the ferric ammonium sulfate to a ferrous compound is accompanied by acidification. French c.s.[7]) even used this to measure the reaction velocity. This certainly cannot account for the observed alkalinization. There is also the possibility of the dehydrogenation of some organic acid as was shown in the previous chapter; however this phenomenon could never be repeated by illuminating the suspension for the second time as in the present instance.

One might even be tempted to regard the phenomenon as a decarboxylation-carboxylation equilibrium which moves in one direction or the other under the influence of light whereas, however, according to Rabinowitch[15]) carboxylation and decarboxylation do not change the average reduction level of the reacting system, in our case the pH changes are accompanied by relatively large changes of the Eh.

Finally it seemed of interest to investigate whether or not carbon-

dioxide is involved in these processes; to this end a number of experiments were carried out, which are described in the following pages.

Experimental material used. 550 mg from second leaf of one month old (June–July), healthy, outdoor grown, oatplant was suspended in 20 ml water. Gold electrode, atmosphere above the suspension nitrogen. Temperature 24°C. (pH determinations were not obtained because of defective apparatus).

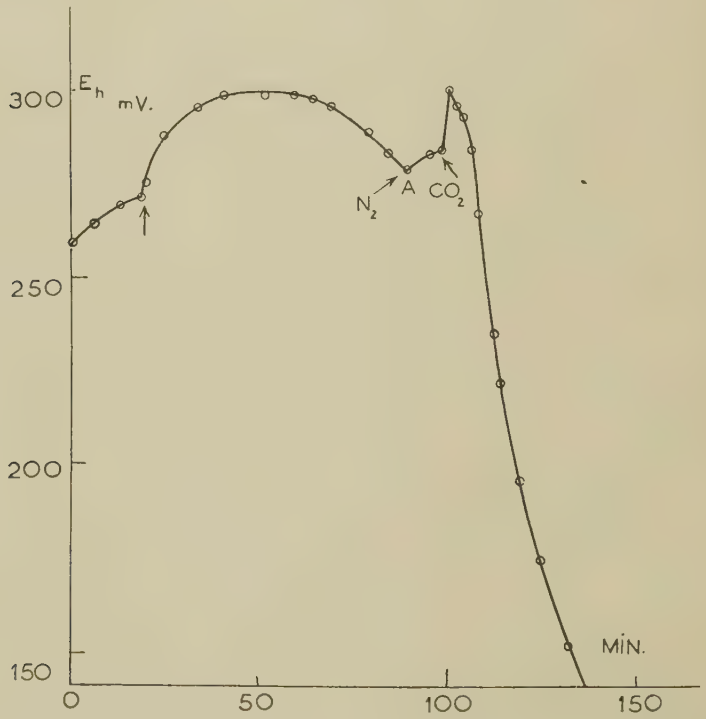

Fig. 6. The air above this suspension has been replaced by nitrogen. The substances determining the potential are slowly oxidized by the oxygen originally present in the suspension and the Eh increases slowly in the dark. On illumination the changes in the Eh are small as well. Bubbling pure nitrogen through the suspension at A has little effect. When however a small amount of CO_2 has been introduced, there is a rapid decrease of the Eh, probably indicating that CO_2 takes part in the photo reduction processes.

During the initial dark period there is a slight increase in the redoxpotential which results from the oxygen originally present in the suspension. On illumination there is a slight increase in the Eh which gradually changes to a decrease. The reactivity of the sus-

pension seemed to be very small and in order to expel the last traces of oxygen which might be present, oxygen free nitrogen was bubbled through the suspension at A. This caused only a slight increase in the Eh. Finally some CO_2 was introduced and as the suspension became more acid, the Eh showed at first a slight increase of 17 mV which was immediately followed by an unexpected sharp decrease of more than 150 mV in 35 minutes. It therefore seemed that the reactivity of the suspension during illumination had been greatly increased by adding a certain amount of CO_2.

In table II the results of a similar experiment are summarized.

TABLE II

The influence of CO_2 on the rate of change of the redoxpotential in a crude chloroplast suspension

Time min.	Eh. mV.	Mean rate of Eh change mV/min.	Time min.	Eh mV	Mean rate of Eh change mV/min.
0	280		55	292	
3	284		60	278	— **2,7**
8	288	+ **0,8**	64	269	
13	290		65	CO_2 introduced	
14	light		67	334	
15	294		70	262	— **18,2**
17	300	+ **1,6**	73	225	
20	305		75	174	
25	311		80	120	— **11,3**
30	313		85	90	
35	313	— **0,1**	85,5	dark	
40	313		86	94	
45	312		90	112	+ **4,0**
50	307		95	120	

Due to the fact that the air above the suspension had been replaced by nitrogen, the increase of the Eh at illumination is only small and after 50 minutes changes into a decrease.

A very short and rapid increase of the Eh (269 mV–334 mV) is caused by the acidification of the suspension when carbondioxide is introduced; this is immediately followed by a sharp decrease at a rate which is nearly 7 times the rate of the decrease just before the addition of CO_2. When at 85 minutes from the beginning of the experiment the light is turned off, the Eh has reached a potential of 90 mV as compared with the normal H electrode.

At that time although the slope of the curve, is decreasing some-

what, it is still very steep and it is to be expected that a much lower potential might have been reached if illumination had been continued.

As already pointed out, there is a sudden increase in the Eh as soon as the light is turned off.

These experiments seem to indicate that carbon dioxide in some way or another takes part in the photoreduction processes. One might object to this on the grounds that in the case of fig. 4 the rate of decrease of the Eh was just as high at illumination as in the present case.

The former experiment, however, was started with air above the suspension and the increase in acidity indicates that a considerable amount of CO_2 must have been produced during the first dark period. In the present case the experiment was started with nitrogen above the suspension and consequently much less CO_2 would be formed.

If this interpretation of the results of these experiments is correct, it is to be expected that when a mixture of nitrogen and carbon dioxide is bubbled through the suspension from the commencement of such an experiment the Eh curve will differ from that in fig. 6, in that there will be an immediate and quick decrease of the Eh as soon as illumination is started. The result of such an experiment is shown in fig. 7 during the first 10 minutes after illumination was started the rate of decrease amounts to 8 mV per min. and finally a potential of —4 mV is reached.

This experiment seems to support the assumption that CO_2 participates in the photoreduction reactions. In order to obtain further evidence it was necessary to determine the Eh and pH simultaneously. To ensure a minimum oxygen tension in the suspension in the next experiment the air was removed by evacuation and replaced by oxygen-free nitrogen. During the second dark period, 50 minutes after the beginning of the experiment, a small amount of oxygenfree carbon dioxide was introduced.

Experimental material used. 750 mg leaf from young oatplant grown in greenhouse (Aug.–Sept.) crushed, filtered and suspended in 10 ml 0,01 N phosphate buffer and 10 ml H_2O.Pt electrode. Temp. 21°C. Initial pH 7,13. Nitrogen above the suspension.

In the first dark period the redoxpotential (A_1B_1) remains almost constant. Immediately after illumination begins, the Eh decreases

rapidly, but the rate of decrease soon slows down and decreases from 4.1 mV per minute in the first 10 min. to 0.6 mV per minute when after 23 minutes the light is turned off. The striking difference when compared with the curve of fig. 7, where nitrogen and CO_2 have been added, is that in the latter case the potential continues to decrease

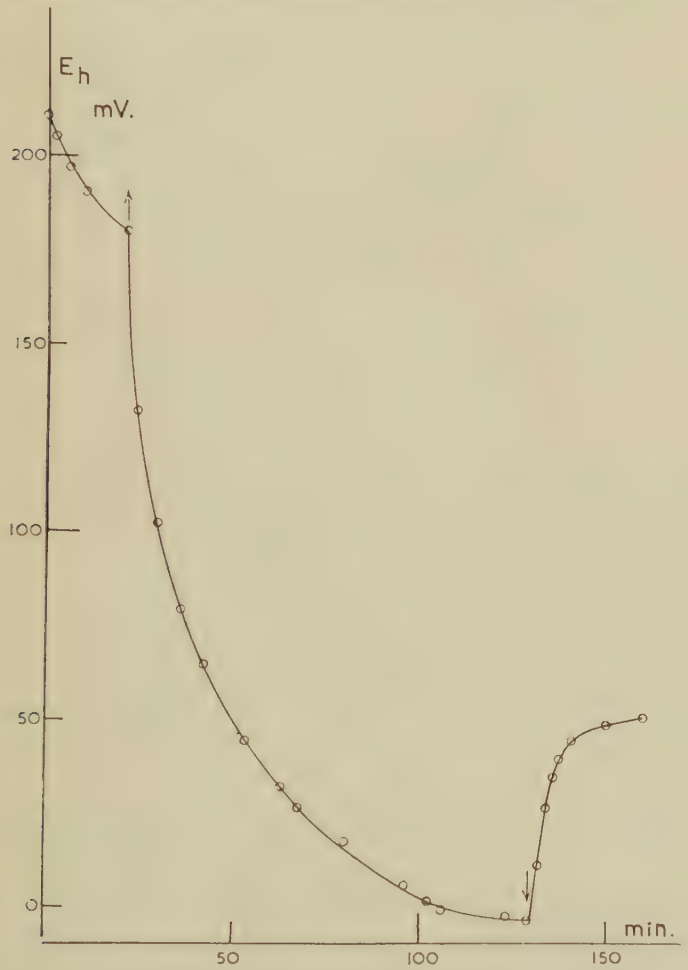

Fig. 7. In this case a mixture of N_2 and CO_2 had been bubbled through the suspension before the experiment was started. On illumination the reduction processes set in immediately and the Eh shows an extremely rapid decrease, finally reaching a minimum of —4 mV. Note the difference on comparison with the conformity of the curves after the addition of CO_2 in the latter case.

for more than 100 minutes until a minimum of —4 mV is reached whereas in the former case after about 50 minutes, a minimum potential of only +270 mV is reached.

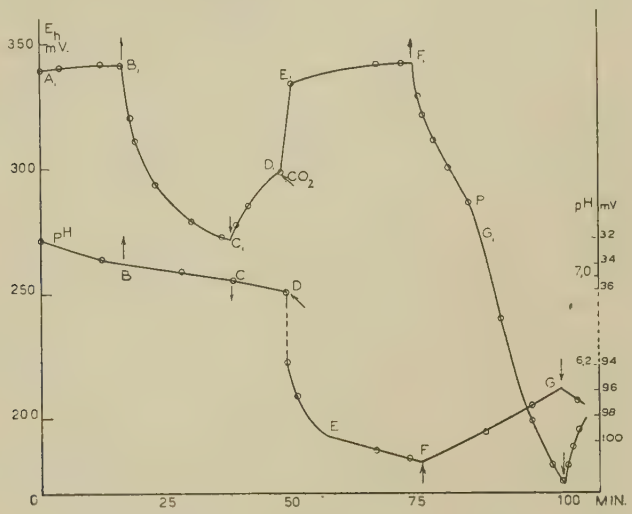

Fig. 8. This suspension has been under reduced pressure to free it from oxygen. The atmosphere above the suspension consisted of pure nitrogen. There is a slow acidification caused by fermentation, the pH does not change on illumination nor on darkening at C. In this anaerobic suspension there is no induction period. When a small amount of CO_2 has been introduced at D, the suspension becomes more alkaline on illumination and the Eh shows a rapid decrease.

Note that the fundamental differences in behaviour at the equipotential points C and G of the Eh curve and of the stretches BC and FG of the pH curve are only caused in the presence of a small amount of CO_2.

During the second dark period the potential increases; this is regularly observed in anaerobic suspensions. The acidification on introduction of CO_2 into the suspension causes the redoxpotential to increase by 35 mV after which it is stabilized at about 340 mV for the rest of the dark period. When the suspension is then illuminated at F_1 the potential decreases rapidly and during the first 15 minutes the curve runs parallel to that of the first illumination period. When however a potential of 285 mV is reached at P, the rate of decrease is accelerated nearly 2.5 times and the redoxpotential has decreased to 175 mV when the light is switched off. It has to be borne in mind that the only difference between the equipotential points C_1 and G_1

is that in the second case the suspension contains a small amount of CO_2, which is obviously responsible for the continued decrease of the redoxpotential.

The pH curve also shows some interesting points. In addition to a very slow initial increase in acidity, most probably due to anaerobic respiration (fermentation), neither illumination nor darkening seem to have any influence on the pH curve (*B–C*).

When CO_2 is added the acidity increases from pH 7.06 to 6.21 and from *E* to *F* the slope of the curve in the dark is exactly the same as that in the previous periods. When the suspension is now illuminated again, there is a decided increase in alkalinity. As soon as the light is switched off, the course of the curve is reversed. On comparing the descending part *BC* of the pH curve in the first illumination period with the ascending part *FG* in the second, it should be remembered that the only difference is a small amount of CO_2 which is present in the latter case and absent in the former.

In a final experiment the redoxpotential and the pH were observed simultaneously and at the end of the experiment the buffer-capacity of the suspension was measured; this made it possible to calculate the amount of CO_2 which had disappeared during illumination.

Experimental material used. 550 mg leaf of healthy oat-plant, grown under conditions of artificial illumination (between Dec. and March) in the greenhouse, were suspended in 20 ml water. Temp. 19°C. Pt electrode. Initial pH 6.80. Nitrogen above the suspension.

After the commencement of illumination a distinct induction period was observed, this was followed by a slow decrease in both the redoxpotential and the pH. This behaviour does not correspond to that observed in the previous experiment probably because in that case all oxygen was removed from the suspension whereas in the present experiment a small amount of oxygen may have been present in the suspension initially. During illumination the CO_2 was added as one ml of a saturated solution of this gas in oxygenfree water. In this way it was possible to supply measured amounts of carbon dioxide. The result was the same as in the other experiments, an increase in the Eh and an increase in acidity (*A -B*). Before the illumination was discontinued the rate of increase in Eh began to

slow down, but it should be noted that in the meantime the reaction had become more alkaline (B-B''). During the second dark period the Eh increased (B_1–C_1), which is a characteristic occurrence under anaerobic conditions after illumination and the reaction became more acid (B''–C). On illuminating the suspension for the second time, the course of the Eh and pH curves is reversed (C–D). The same thing happens during the third (D–E) and fourth dark periods

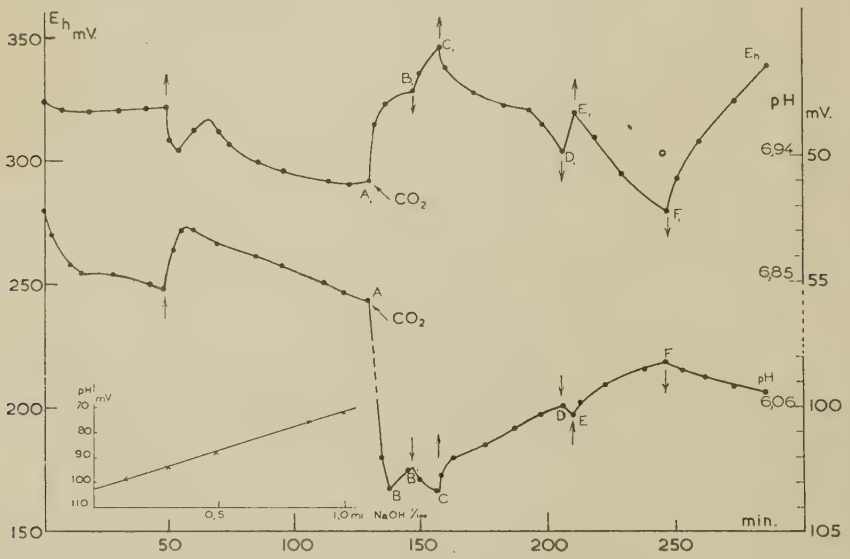

Fig. 9. Though the air above the suspension had been replaced by nitrogen the suspension still contained some oxygen at the beginning of the experiment. Hence a distinct induction period is observed on illumination. During the first illumination period the suspension slowly becomes more acid, whereas the Eh only shows a slight decrease. After the introduction of a small quantity of CO_2 the suspension alternatively becomes more alkaline during illumination and more acid in the dark. The Eh changes are also much more pronounced. Both facts combined make the participation of the CO_2 in the photoreduction processes highly probable.
In the left corner of the fig. the titration curve of the suspension has been represented.

(F–G), whereas in the third illumination period (E–F) the Eh decreases and the reaction becomes less acid.

This case is particularly interesting as it enables a distinction to be made between the initial induction period and the photoreduction of the CO_2. The sudden decrease in acidity and a corresponding

decrease of the Eh when a suspension is illuminated for the first time is characteristic for the induction period, this coincides with the photo-oxidative destruction of the ascorbic acid.

The decrease in acidity after addition of the CO_2 is less explosive and is not followed by a decrease, but increases steadily until the light is switched off. In the subsequent dark period the suspension slowly becomes more acid. Further evidence for the different origin of the decrease in acidity is the fact that in the previous experiment (fig. 8) there was no induction period at all and a very distinct decrease in acidity during the illumination period after the addition of the CO_2. In the present case special stress is laid upon the fact that before the addition of CO_2 the suspension becomes more acid during illumination, whereas after the addition of CO_2 it becomes more alkaline. From the composable results of these experiments, made with *Avena* plant material of different origin and at different seasons, it may be concluded that in these crude chloroplast suspensions under anaerobic conditions carbon dioxide participates in the photoreduction reactions.

Calculation of the amount of CO_2 which disappeared during illumination of the suspension (fig. 9). When the suspension was titrated with 0,01 N NaOH, 1 ml caused a decrease of the potential of the glass electrode of 28,3 mV. This equals $\frac{44}{200}$ = 0,22 mg CO_2 or 7,8 μg CO_2 per mV.

The suspension had been illuminated for 93 min. during which the decrease in acidity, expressed in millivolts, amounted to 6,5 mV or 4,2 mV in one hour.

The total amount of CO_2 disappearing from the 8 ml of the chloroplast duspension in one hour is 4,2 × 7,8 = 32,8 μg.

According to previous investigations the mean quantity of CO_2 assimilated per 100 cm^2 leaf surface of healthy oatplants amounts to 10 mg.

In the present case the surface of 550 mg of leaf was about 30 cm^2 and this would have assimilated 3,0 mg CO_2 in one hour.

These 550 mg leaf were suspended in 20 ml water, 8 ml of which were put in the Warburg flask. Normally this quantity would have assimilated $\frac{8}{20}$ × 3,0 = 1,2 mg CO_2.

During illumination of the suspension the decrease in acidity, equalled 32,8 μg CO_2 and assuming that this has been assimilated, this amounts to 32,8/1200 × 100 = 2,7% of the CO_2 which would have been assimilated under normal circumstances.

Discussion. Though the general course of the Eh and pH changes during periods of darkness and illumination in crude chloroplast suspensions containing oxygen needs no further explanation, the phenomenon of the so called induction period deserves a closer examination.

The induction period and the role of ascorbic acid in photosynthesis. It seems that the term "induction period" covers a wide range of different changes which have to occur before photosynthesis can proceed at normal speed.

It may be questioned as to whether the induction periods observed in the pH and Eh curves of these crude chloroplast suspensions bear any relation to the induction phenomena observed in intact plants.

According to van der Veen[19]) "immediately after illumination different processes, which make up photosynthesis, are initiated, partly independent of each other." The induction phenomenon is a complicated series of reactions of which an increased initial CO_2 uptake as observed by Aufdemgarten[1]) and van der Veen[19]) is one of the components, according to the same author.

The temporary decrease in acidity observed in the present experiment might be caused by this initial increased CO_2 uptake as well as by decarboxylation of organic acids, primarily ascorbic acid, though other acids might play a similar role as many plants contain various organic acids of which malic and citric acid are the most common[15]). p. 362).

In the present investigation it was shown that when a suspension of crushed *Avena* leaves is illuminated, the temporary decrease in acidity coincides with the disappearance of the ascorbic acid originally present. Furthermore the addition of small amounts of ascorbic acid to the suspension intensifies the observed induction phenomena, whereas the characteristic points of the Eh and pH curves in the latter case correspond exactly with those in the former.

It therefore seemed rather logical to ascribe a definite role to ascorbic acid in the induction phenomena observed when a crude chloroplast suspension is illuminated for the first time. It is also to be expected that in the intact plant a highly reactive substance such as ascorbic acid takes part in the oxidation reduction processes. From experimenths with various waterplants, whose photosynthetic activity is stimulated by ascorbic acid and from in vitro model experiments

Bukatsch[4]) concludes that ascorbic acid plays a prominent part as an auxiliary redoxsystem in photosynthesis.

In the present experiment where aerobic conditions were used the ascorbic acid is ultimately completely destroyed by photo-oxidation and consequently the phenomenon could not be reproduced during a second period of illumination.

In intact leaves cell structure limits the free entrance of oxygen and regulates its diffusion into the cells: this prevents the total destruction of the ascorbic acid and makes repeated use, in the oxidised and reduced form, possible.

Surveying the situation in a living cell at the end of a dark period a certain amount of carbon dioxide as well as oxygen, will be present among the cell constituents, the concentration of oxygen present being the resultant of the oxygen uptake by respiration and the renewed supply by diffusion.

According to Neish[14]) ascorbic acid is approximately uniformly distributed between the chloroplasts and other parts of the leaf cells. It will be present partly as such and partly as dehydro-ascorbic acid, the equilibrium between both components, i.e. the redoxpotential, depending on the pH, oxygen tension and the presence of other oxidation reduction systems.

According to Ball[2]) the redoxpotential of the ascorbic acid-dehydroascorbic acid system lies between $E_0 = 0.329$ mV for pH 1 and $E_0' = 0.057$ mV for pH 7. At an Eh of $\pm$ 250 mV, which is generally observed in the crude chloroplast suspensions, the equilibrium will be shifted towards the side of the dehydroascorbic acid. As soon as illumination is started actual photosynthesis, i.e. the photoreduction of CO_2, cannot proceed unless a certain redoxpotential is reached; this means that the greater part of the oxygen has to be hydrogenized first and at the same time, the ascorbic acid-dehydroascorbic acid equilibrium has to be moved in the direction of ascorbic acid. Both processes will temporarily monopolize the reducing power of the photosynthetic apparatus and will be responsible, partly or totally, for the delay of photosynthesis at the beginning of an illumination period, e.g. for the so called induction period. When an illumination period is followed by a period of darkness it is understandable that it takes some time to reach the same condi-

tion as prevailed before illumination. Oxygen will rediffuse into the centres previously photosynthetically active, the ascorbic acid-dehydroascorbic acid equilibrium will be moved in the direction of the more oxidized component, which will certainly be accompanied by a diffusion of both components from places of higher to those of lower concentration.

In this connection it is of interest to note that the black silver deposit in chloroplasts resulting from Molisch's silver nitrate test often shows a granular pattern, which might indicate that though the mean concentration of ascorbic acid in chloroplasts and cell sap is approximately the same, there are local differences in concentration separated by microscopical distances inside the chloroplasts.

An important fact is the pronounced influence of illumination on the ascorbic acid content or leaves of different plants, as observed by Somers c.s. [18]). They showed that the ascorbic acid content of a leaf *increased* in light and *decreased* in the dark, which is a strong evidence for the effect of light on the ascorbic acid-dehydroascorbic acid equilibrium as postulated above *). Van der Veen [19]) showed that the duration of the preceding dark period had considerable influence on the shape of the induction curve and that the longer the dark period has lasted, the slower was the return to maximal assimilation.

He assumes the existance of some unknown factor, which has to be activated by illumination.

In the present experiments it is supposed that in the dark period oxygen rediffuses into the nuclei of photosynthetic activity and that the ascorbic acid-dehydroascorbic acid equilibrium returns to a less reduced state, both changes taking time to reach the original dark equilibrium levels.

During the induction period the oxygen and the dehydroascorbic acid have to be hydrogenated, before the actual photoreduction of CO_2 can set in, therefore it is clear that the duration of the induction period, within certain limits, will depend on the duration of the preceding dark period **).

*) It is emphasized that with the method of Nelson and Somers [14a]), Somers used to determine the ascorbic acid, only the reduced form is determined whereas the dehydro ascorbic acid does not affect the 2–6 dichlorophenolindophenol solution.

**) There is no reason to suppose that these are the only factors which may cause or contribute to the induction phenomena. In this connection the reader is reminded of the experiment on page 183 fig. 12 of the second paper of this series.

If this interpretation of the induction period is correct, it is to be expected that it will be absent from suspensions under anaerobic conditions. In that case the ascorbic acid equilibrium has moved to complete reduction and no oxygen is present to disturb the hydrogen transfer from its normal path.

In this connection it is interesting to note that Gaffron[10]) did not observe the usual induction period with *Scenedesmus*, when this alga had been exposed to a prolonged anaerobic period. Also in the case of fig. 8 in the present investigation, where the crude chloroplast suspension was first placed under reduced pressure, to get rid of oxygen and then kept under nitrogen, not a trace of an induction period could be observed.

Since in both cases the observations made on totally different material gave the same results it is highly probable that the same phenomenon is involved in each case; this strongly supports the assumption postulated above.

Since the ascorbic acid -dehydroascorbic acid equilibrium is moved in the direction of ascorbic acid during illumination, it is to be expected that the Molisch reaction will proceed more rapidly in light then in darkness. This has indeed been observed, in the first instance by Gautheret[11]), who claimed that the Molisch reaction occurs only in light; Giroud[13]) observed that light markedly accelerates the reaction, but that it also proceeds in the dark.

Steemann Nielsen[17]) has shown that the reestablishment of the original dark conditions is markedly accelerated by higher temperatures: van der Veen[19]) confirmed this observation.

This phenomenon might be ascribed, not only to a positive temperature coefficient of the chemical reactions eventually involved, but also to the rate of diffusion of oxygen into the cells as well as the equalizing, by diffusion, of the concentrations of ascorbic acid and dehydroascorbic acid, both being directly proportional to temperature.

With regard to the role ascorbic acid plays in photosynthesis the present author is of the opinion that this substance is preeminently suited as an intermediate oxidation reduction system between water and carbon dioxide. According to Rabinowitch[15]), photosynthesis might require one or several such intermediates, which are

concerned with hydrogen transfer (or electron transfer) between adjacent oxidation reduction systems. The existance of such intermediates makes a topochemical mechanism having widely divergent redoxpotentials in specific nuclei at microscopic distances possible; such a system may account for the absence of, or even be indispensible for the prevention of backreactions. The objection that the potential of such an ascorbic acid redoxsystem at the pH generally prevailing in the cell sap, lies far above the potential necessary for the reduction of carbon dioxide, will be dealt with in the following pages.

Redoxpotential and pH changes as influenced by carbon dioxide under anaerobic conditions. The principal result of the pH and Eh measurements on the crude chloroplast suspensions under anaerobic conditions and during illumination are the decrease in acidity and the influence of carbon dioxide on the redoxpotential.

The question may be raised as to whether the decrease in acidity has not been caused by partial photo-oxidation of ascorbic acid, which is reversed in the subsequent dark period. That this is not the case is demonstrated by the results of the experiments depicted in figs. 9 and 8. The curve in fig. 9 shows a typical induction period due to photo-oxidation of ascorbic acid. The first period of illumination lasted 80 minutes which was certainly adequate for the destruction of all the ascorbic acid originally present in the suspension. During illumination the suspension gradually becomes more acid. At the point where the CO_2 is introduced the curve does not show any tendency to reverse its course; a few minuter later however, when the acidification due to the addition of CO_2 has settled down, there is a sharp turning point, indicating that acidification has been replaced by alkalinization, a process which continues so long as illumination lasts. The reverse process takes place when the light is switched off. It is obvious that after the introduction of CO_2 a different process is initiated.

In fig. 8 an induction period is not observed in the pH curve, there being no detectible response either to illumination or dark periods. The slow and steady acidification which takes place, independent of light or darkness, might be due to some kind of internal respiration or fermentation.

After the introduction of carbon dioxide the picture changes fundamentally and the acidity is markedly influenced by illumination and darkening. The pH change caused by the CO_2 certainly cannot be held responsible for this change in behaviour of the suspension; these pH changes are relatively small, 0.7–1 pH units, and occur in the region between pH 7 and 6, which is a normal acidity for many plants.

One might object that the pH changes under influence of illumination are very small and therefore liable to errors in measurement. However the regular sequence of the different points of the curves and the agreement between the results obtained with plant material of different origin and at different seasons indicate the validity of the observations.

The only possible explanation of the increase of the pH during illumination is that it is caused by the disappearance of CO_2 caused either by the formation of a complex (CO_2) or by its actual participation in the photoreductive process.

Fixation of CO_2 by complex formation has frequently been observed in intact plants in the dark [15]), (p. 200–209) and it seems logical to suppose that it also proceeds during illumination.

The experiments of Ruben c.s. [16]) warrant the supposition that most, if not all, of the carbon dioxide in the (CO_2) complex is contained in a carboxylgroup, so a marked decrease in acidity is not to be expected. Moreover the formation of such a complex will not be accompanied by a decrease of the Eh by more than 200 mV as is the case in figs. 7 and 8.

The fact that in all cases the alkalinization of the suspension is accompanied by an important decrease in the redoxpotential, which is absent in the absence of carbon dioxide, makes it highly probable that under the circumstances of these experiments CO_2 partakes in the photoreduction processes.

Though the quantity is small and amounts to only a few percent of the normal assimilation of the intact leaf, it can be repeated by alternate illumination and darkening which points to the possibility that this phenomenon concerns an integral part of the photosynthetic apparatus.

One might be tempted to regard the phenomenon as a reversible equilibrium, the balance of which depends on illumination; a simple carboxylation-decarboxylation equilibrium however, corresponds

neither with the observed pH changes nor with the large decrease in the redoxpotential.

Though the behaviour of succulent plants bears a striking resemblance to that of these suspensions, e.g. that during the night acidification prevails which is replaced by a more alkaline reaction in daylight, there seems to be no reason to suppose that in our case organic acids play a role of similar importance.

Finally the objection might be raised that at redoxpotentials prevailing in the chloroplast suspensions photoreduction of CO_2 is out of question. This however is not a special problem in this case, but a problem of photosynthesis in general. There is no reason to suppose that in a thin leaf surrounded by air the extremely low redoxpotentials, which would be necessary for the photoreduction of CO_2 as such will exist. This problem and the role the energy rich phosphate bonds may play in this connection has been dealt with in the second paper of this series. On the other hand one might consider the present experiments as a proof that photoreduction of CO_2, in an activated state or as part of a complex, is possible at the relatively high potentials prevailing in these crude chloroplast suspensions.

There seems to be a close relationship between the behaviour of these crude chloroplast suspensions and the experiments of Gaffron [9]) using anaerobically incubated Scenedesmus. In both cases oxygen is not produced during the photoreduction of the CO_2, which is the main difference composed with normal photosynthesis. In both cases the induction period disappears after prolonged anaerobiosis (Gaffron [10]) and in the experiment depicted in fig. 8 of the present paper).

Applying van Niel's concept [15a]), that all forms of bacterial photosynthesis are to be regarded as light induced hydrogen transfers from various hydrogen donors to carbon dioxide as the common hydrogen acceptor, to photosynthesis in general, his well known equation

$$CO_2 + 2\,RH_2 = ((CH_2O)) + H_2O + 2\,R''$$

may give a reasonable explanation of the behaviour of the crude chloroplast suspensions.

In that case one or more of the cell constituents present in the suspension might function as RH_2 in place of the H_2O as occurs in normal photosynthesis. It would be highly interesting to investigate

the effect of hydrogen and of hydrogen donors like glucose on these anaerobic photoreduction processes along the lines of the important work of G a f f r o n on *Scenedesmus*.

The observed decrease of the Eh on illumination after the introduction of CO_2 might be ascribed to the formation of a primary assimilation product of high reducing power, of the type RCHO. The increase in acidity and the increase of the Eh in the dark periods following illumination might indicate that the reverse of the above mentioned process takes place, e.g. the oxidation of the primary assimilation product by the dehydrogenated organic substances which in the foregoing period have functioned as hydrogen donors, a process to some extent analogous to fermentation.

Though it is premature to express any definite opinion with regard to the nature of the observed phenomena in these crude chloroplast suspensions, the author is inclined to apply to the present case the interpretation of F r a n c k and G a f f r o n[6]) "that in the reduced state of the plant system carbon dioxide reduction proceeds by the way an oxido-reduction as in the purple bacteria which are able to reduce CO_2 at the cost of hydrogen from organic hydrogen donors".

Acknowledgement. The author is very much indebted to Dr. W. R. C. H a n d l e y of Oxford University, who has kindly corrected the manuscript grammatically and who has made a number of valuable suggestions to improve the text of this paper.

Summary

The changes in the pH and in the redoxpotential of crude chloroplast suspensions were observed during illumination and whilst in the dark, under aerobic and anaerobic conditions.

In the dark, under aerobic conditions, the acidity generally increases slowly, due to CO_2 production caused by residual respiration of the suspension.

When illumination is started, a more or less pronounced induction period is observed, which is accompanied by a temporary decrease of acidity and a less pronounced decrease of the Eh. These induction periods are far less pronounced or are not observed at all, when the suspension is illuminated for the second time.

It was shown that the decrease in acidity during the induction period coincided with the disappearance of the ascorbic acid normally present in the leaf. By addition of a small amount of ascorbic acid to the suspension the

typical phases of the induction period were accentuated. Under strict anaerobic conditions no induction period was observed.

As this has also been observed in intact plants, there seem to be certain resemblances in both cases.

It is assumed that one of the factors causing retardation of actual photosynthesis when illumination is started may be that the ascorbic acid–dehydroascorbic acid equilibrium first has to be moved in the direction of ascorbic acid, dehydroascorbic acid being much more easily hydrogenated than carbon dioxide.

With regard to the pH and Eh changes under anaerobic conditions in the dark it was observed that the acidity increases slowly, probably the result of fermentation; also the changes in the Eh are small.

The slow acidification of the suspension continues during illumination, whereas there is a somewhat larger decrease of the Eh, varying between 25 and 70 mV.

However, as soon as a small amount of CO_2 has been introduced the change of the pH is reversed during illumination, the suspension becoming more alkaline and at the same time the redoxpotential decreases very quickly, in some cases even to the extent of about 200 mV.

Both facts combined point to an active participation of the CO_2 in the photoreduction processes in these crude chloroplast suspensions. The quantity of the CO_2 which disappears during illumination however is small and only amounts to 2–3% of the CO_2 assimilated by the corresponding surface of a normal leaf in the same time.

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BIOSYNTHESIS OF AMINO ACIDS ACCORDING TO SOIL FERTILITY

I. TRYPTOPHANE IN FORAGE CROPS [1] *)

by V. L. SHELDON, WM. G. BLUE and WM. A. ALBRECHT

University of Missouri, Columbia, Missouri U.S.A.

Our complete dependence on plants for their synthesis of many amino acids calls for our best efforts to explain the biosynthesis of these vital substances. The two fundamental concepts, namely, (*a*) that proteins are composed of amino acids, and (*b*) that the construction of these protein particles occurs only when all the requisite building stones are present, includes the major share of our knowledge about protein synthesis. Any elucidation of the mechanism by which the amino acids are formed would seemingly call for study of the essentially and of the functions of the inorganic elements catalizing these synthese reactions in plants and other life forms.

The service of the various soils, as they may, or may not, deliver adequate supplies of the inorganic nutrient elements, emphasizes further the need for studies integrating the elements of soil fertility with the resulting organic chemical pattern within feed or food crops. Here, then, is the suggestion of the basic fact that our nutrition, and resulting health, ultimately depend upon the soil and the climatic forces that determine it. The development of the divergent soils from the various rocks according to rainfall, temperature, and other meteorological components of climate has definitely established the control of the soil by the climate. We can then reason that through its inorganic contribution to the plant, the soil controls the nutritive quality of food. In turn, this quality of food controls our health.

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The leaf, rather than the stem of the green plant provides a biosynthetic factory wherein we may view the diversity in the array of the organic products [5]. The materials entering the plant from its environment are, for the most part, inorganic elements of the most simple character. By proper manipulation of these substances the plant can build a large variety of molecules of widely differing degrees of complexity.

Previous studies. Protein synthesis, conceivably, begins with the transformation of the carbon chain. The companionship of magnesium and phosphorus in the glycolytic system assumes added importance when viewed in this new light of synthesis. Calcium and potassium interplay for the accompanying reduction of the nitrate. Iron, magnesium, copper, molybdenum, and boron all exert their respective influences. Zinc has been shown to be required for tryptophane synthesis [11]. The rather extensive literature on protein synthesis has been reviewed at different times by McKee [2], Nightingale [3], Chibnall [1], and Wilson [12]. Bjorksten believes that amino acid synthesis involves the condensation of the enol form of pyruvic acid with an acid amide. The resulting product breaks down to form an organic acid and aminoacrylic acid. The latter condenses with other compounds. Petrie [4] believes that in plants the tricarboxylic acid cycle may be operative in the production of succinic and α-ketoglutaric acids. These may be converted into their respective amino acids by transamination, and serve to store excess nitrogen in the form of their amides, asparagine and glutamine. The primary question involves the reduction of nitrates to nitrites and ammonia. This reaction seems to be coupled with the oxidation of carbohydrates.

That a metabolic block, which would increase the content of free acids, including the amino, might occur when the soil-borne elements were not present in sufficient amounts, has recently been shown by the work of Steinberg, Bowling and McMurtey [7]. They indicate that free amino acids in leaves of plants showing symptoms of low mineral supply increased with nitrogen deficiency by 32%; phosphorus 48%; potassium 58%; calcium 120%; magnesium 283%; and boron 27%. Tisdale *at al.* [10] reported the methionine and cystine contents of two strains of alfalfa indicating that the sulfate in the substrate influenced the concentration of

these two organic acids in the alfalfa forage. The authors [6]) showed that optimum concentration of sulfate for methionine production by alfalfa was 64 p.p.m. and by soybeans 96 p.p.m. They further indicated that the application of 200 pounds per acre of Flowers of Sulfur to a planosol soil of the Southwest Missouri prairie region almost doubled the concentration of methionine in the dry Sudan grass. The work reported herewith is a similar study in which there was determined the tryptophane concentration in various forages grown on various cultural media in the greenhouse.

Experimental Methods. The experimental approach consisted of the critical assays for tryptophane in plant samples grown in both solution and colloidal clay cultures. Soybeans and alfalfa were grown on white quartz sand offered the nutrient solution given in Table I.

TABLE I

Nutrient solution used in quartz sand cultures	
Element	Full Nutrient (p.p.m.)
K	547.00
Ca	400.00
Mg	96.00
N	24.80
P	124.00
S	128.00
Fe	34.06
Mn	1.10
B	1.08

Magnesium, manganese, boron, and iron were withheld as individual omissions only. Soybeans were also grown on electrodialyzed colloidal clay and certain silt mineral mixtures. The treatments and results for these are given in Table II. Redtop was grown on colloidal cultures with high, medium, and low calcium each coupled separately with high and medium phosphorus.

Random samples of the plants produced under these environmental conditions were harvested, dried at 70°C, ground to pass through a 60-mesh sieve and stored in air-tight glass containers for assay in the laboratory. The tryptophane was determined on the hydrolyzed plant materials by the microbiological procedure of

TABLE II

Concentration of tryptophane in soybean hay grown on colloidal Putnam clay and magnesium mineral mixtures *)

Culture Composition	Tryptophane mg/g	Total Nitrogen %
10 g Wyomingite, 50 g H-clay, and 0.5 mmols. H_3PO_4	0.47—0.50	1.94
10 g Glauconitic dolomite, 50 g H-clay, and 0.5 mmols. H_3PO_4	0.66—0.90	1.40
50 g H-clay, 25 m.e. Ca, 10 m.e.Mg, and 0.5 mmols. H_3PO_4	1.06—1.09	1.56

*) These plants grown in greenhouse culture by H. C. Turley.

Stokes and Gunness [8] [9] using *Lactobacillus arabinosus* 17–5 as the assay microbe.

Results. Indol-3-acetic acid has frequently been associated with the boron supply from the soil. There is the suggestion in the data in Table III of a possible relation between this element and the con-

TABLE III

Concentration of tryptophane in alfalfa and soybean hays according to amounts of boron offered in the nutrient solutions

Soybean Hay		Alfalfa Hay	
Boron Offered p.p.m.	Tryptophane mg/g	Boron Offered p.p.m.	Tryptophane mg/g
0	1.38	0	1.27
0.27	1.89	0.22	1.36
1.28	3.10	0.44	2.17
		1.08	2.55

centration of the indol amino acid-tryptophane-in both the alfalfa and soybean hays. Even though the enzymatic function of boron remains quite obscure, these data substantiate the conclusions of Briggs, namely, that an interruption in the synthetic processes just prior to protein formation occurs when boron is in low supply.

In an effort to determine the magnitude of tryptophane synthesis when the elements commonly supplied by the soil are in low amounts magnesium, boron, manganese, iron, calcium, and phosphorus were each withheld separately from the growth medium. The results of these modifications of the plant environment, recorded in Table IV, show the pronounced variation in the concentration of this essential amino acid in soybeans and alfalfa as legumes and in redtop, a grass,

TABLE IV

Concentrations of tryptophane in alfalfa, soybean, and redtop hays according to inorganic nutrients precent in the substrate

Plant	Treatment	Tryptophane mg/g
Soybeans	Mg withheld	1.80
Soybeans	B withheld	1.89
Soybeans	Full nutrient	3.10
Soybeans	Mn withheld	1.93
Soybeans	B withheld	0.57
Soybeans	Fe withheld	1.76
Soybeans	Full nutrient	2.10
Soybeans	Wyomingite	0.47—0.50
Soybeans	Glauconitic dolomite	0.66—0.90
Soybeans	Exch. Mg. on clay	1.06—1.09
Alfalfa	Mn withheld	1.47
Alfalfa	B withheld	1.15
Alfalfa	S withheld	2.64
Alfalfa	Fe withheld	1.74
Alfalfa	Full nutrient	2.80
Redtop *)	High P, High Ca	2.65
Redtop	High P, Medium Ca	2.21
Redtop	High P, Low Ca	2.09
Redtop	Medium P, High Ca	2.38
Redtop	Medium P, Medium Ca	1.88
Redtop	Medium P, Low Ca	1.38

*) Grown on colloidal clay culture.

according to the corresponding shortage of these elements of soil fertility. Where magnesium was withheld from soybeans grown in solution, the tryptophane content was reduced from 3.1 to 1.8 milligrams per gram of dry forage. In studies of some magnesium minerals from which this element could be weathered at varying rates by the acid colloidal clay, the tryptophane content was lowered in proportion to the magnesium made avalable from that reaction. The concentration of this amino acid in the case where Wyomingite was offered was only one-half of the value of that where the magnesium was readily exchangeable on the clay as shown in Table II. Magnesium from the dolomite was more available and expressed itself through increased synthesis of tryptophane by the plants. These data carry the suggestion that either less carbohydrate was

built by photosynthesis or less was respired to yield a critical linkage in the tryptophane molecule. The enzymatic formation of the indol ring could require magnesium as the activating cation at some stage of its synthesis.

Where boron was withheld, the tryptophane in the forage was always substantially lower. When manganese was omitted from the solution offered these alfalfa plants, the concentration of this amino acid in the hay was reduced 50% below that from the full nutrient solution. When sulfur was not supplied little if any reduction in tryptophane formation occurred. Both calcium and phosphorus seemingly promote production of this essential amino acid, if we are to judge from the data for the redtop in Table IV. This grass was grown on two levels of phosphorus each coupled on two levels of phosphorus each coupled with high, medium, and low applications of calcium. On both levels of phosphorus, the tryptophane in the forage was increasingly higher with increasing concentration of calcium. The tryptophane values for the plants receiving high phosphorus treatments were significantly above those given medium amounts of phosphorus. While the function of the elements in tryptophane synthesis is far from clear, general considerations point to the part these ions might play. Phopshate, for example, is the key ion in intermediate metabolism. It must be supplied by the soil in quantities commensurate with the needs of these carbohydrate transformations. Phosphate serves not only to supply sufficient energy to maintain the living state, but also to aid in the biosynthesis of such molecules as may be precursory to tryptophane. Also, we have the suggestion that at least zinc, boron, manganese, and now calcium, phosphorus, and magnesium are involved. In view of the many roles of these soil-borne nutrients in the intermediate metabolic pool, precise study of these enzymatic relations is strongly indicated for the future work.

Discussion. In the physiology of an organism we have regularly recognized the necessity of considering the inorganic environment in attempts to explain any performance by it. Yet the soil, as the source of nearly all the required nutrient elements remains much of an unknown. The measurement of the delivery of the inorganic elements in the composition of the burnt plant material, as the only effect of the soil, has been an unfortunate, though prevalent, criterion of the contribution by the soil factor of the environment. This little appre-

ciation of the soil has persisted even when the ecological array of plant species suggests that plants must be of diverse organic quality as they evolve on the diverse soils.

Consideration of the physiological roles of the inorganic nutrients has led us in this study to picture them active in the enzymatic mechanisms of biosynthesis. This process, in which the simple carbohydrate is converted into the protoplasmic protein by the help of the soil-borne elements, demands a more detailed study of the plant enzymes containing these inorganic nutriens. The biocatalysts performing in the construction of the protein include those functioning in the intermediate metabolic pool. The key to amino acid synthesis would seem to lie in a study of enzymes containing, or activated by, the soil-borne nutrients wherein these biocatalysts enable the formation of the residual structures precursory to each amino acid molecule.

Visual symptoms of plant deficiency need not be evident to prove deficiencies in the health, or vigor, of the organism. Deficiency is a matter of degree. Plants, like animals, may become decidedly inferior in quality long before showing outward signs of such. It is in this broad range between optimum and deficiency that such biochemical assays of quality assume greater importance. It would seem that any biological assay of soil fertility must ultimately measure molecules as the reactants in biosynthesis, and not merely the delivery of ash in the forage. The whole biotic pyramid may well be viewed as the simple combinations of colloidal particles according to enzymatic specificity to yield the vital cellular constituents according to the fertility of the soil.

Summary

A microbiological assay for tryptophane, as a required amino acid for the diet of the white rat, in alfalfa (*Medicago sativa*), soybean (*Soja max*), and redtop (*Agrostis stolonifera major*) hays, showed that this organic substance of particular food value varied widely according to the inorganic composition of the substrate upon which the plants synthesizing it were grown. The formation of tryptophane was found to be proportional to the available boron when this anion was the limiting element in the culture solution. Tryptophane synthesis was decreased when magnesium, boron, manganese, and iron were withheld from solutions offered alfalfa and soybeans. Increasing the calcium increased the production of this amino acid. The enzymatic pattern of biosynthesis revealed itself pointing out some

possible reasons why such diversity in the components of the plant protein might be expected. The effects of the inorganic nutrient elements were shown to be of the same kind whether the plants were grown in nutrient solution or in colloidal clay.

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NOUVELLE MÉTHODE DE DOSAGE DES ÉLÉMENTS MINÉRAUX FERTILISANTS DU SOL PAR LES AZOTOBACTER

par WANG T. L.

Institut Pasteur (Laboratoire de Microbiologie technique)

Pour apprécier le fertilité du sol, les laboratoires agronomiques disposent de deux groupes de méthodes d'analyse: Les dosages chimiques des éléments dits „assimilables" et les analyses biologiques dont les réactifs ne sont autres que les végétaux eux-mêmes ou des microorganismes. Parmi ces derniers, les plus fréquemment utilisés sont les *Azobacter* et *Aspergillus* (Bortels, Van Niel, Curie, Sacket, Steward, Ziemecka, Winogradsky, Mulder, Loureire, Efendiever, Manoquier, Tamaya ....). On admet que l'activité microbienne est en relation étroite avec la croissance des végétaux, donc avec l'état de fertilité du sol, surtout dans le cas d'une carence minérale (Chouchak, Waksman, Starkey, Henkele-Vian, Lees ....). Cependant les techniques biologiques proposées sont surtout qualitatives et deviennent compliquées et délicates quand on les utilise pour effectuer un dosage. Nous proposons ici une technique d'analyse quantitative biologique des éléments minéraux assimilables de la terre.

I. *Principe de la méthode.* Elle est basée sur l'exigence des *Azotobacter* en éléments minéraux pour leur nutrition et pour la production de leurs enzymes.

En effet, nous savons que les *Azotobacter* produisent une substance noire en attaquant le benzoate de Na [2]). Or la production de celle-ci est proportionnelle à la quanité d un élément minéral pris en particulier; il suffit donc de doser la substance noire formée pour évaluer la quantité de l'élément à étudier.

Pour cela nous préparons un milieu an benzoate de sodium privé

d'un élément choisi, par exemple le phosphore. Ce milieu, réparti dans des fioles, est additionné de quantités croissantes de P sous forme de phosphate de potassium. On ensemence avec *Azotobacter* et on incube à 36°C. Après une quinzaine de jours, on dose colorimétriquement la substance noire formée. On trouve dans ce cas, pour certaines concentrations, des points qui se situent sur une ligne droite proportionnelle aux quantités de phosphores ajoutées. Ce qui est valable pour P se révèle également valable pour les autres éléments tels que K, Ca, Mg, Fe et Mn.

Si, dans ce milieu privé d'un élément, on ajoute une certaine quantité de terre, le dosage de la substance noire après l'incubation permet d'évaluer la quantité de cet élément, assimilable par les *Azotobacter*, apporté par la terre, par comparaison avec la gamme étalon précédente.

II. *Technique.* 1. Préparation de la terre. Prendre la terre à étudier, la sécher à la température du laboratoire. Eliminer les cailloux et les autres résidus. Pulvériser la terre aussi finément que possible et la conserver dans des bouteilles bien bouchées. Peser sur une balance de précision au moment de l'emploi.

2. Préparation du milieu. Milieu type:

$CaCl_2$	0.075 g
PO_4H_2K ou PO_4H_2Na	0.800 „
SO_4Mg	0.250 „
NaCl	0.500 „
SO_4Fe	0.015 „
SO_4Mn	0.008 „
Eau distillée	200 cc

Ce milieu doit être dilué à raison de 50 cc per litre d'eau distillée. On y ajoute en outre 2 g de benzoate de sodium par litre. Le pH est ajusté à 7.45. Stériliser à 120°C pendant 20 minutes. L'alcali utilisé pour amener le milieu à 7.45 est soit NaOH, pour le cas où on veut priver le milieu de K, soit KOH pour les autres éléments. Le phosphate utilisé varie avec l'élément à éliminer.

Si l'on élimine un élément quelconque de ce milieu, celui-ci est alors utilisable pour le dosage *).

*) A noter qu'il n'est pas nécessaire d'avoir des produits absoluments purs, au sens strict du mot, puisque la gamme étalon est réalisée avec les mêmes produits; le dosage se fait donc par différence.

3. Préparation de la suspension azotobactérienne. Elle doit elle même être privée de l'élément à doser.

On encemence *Azotobacter chroococcum* sur le milieu type, gélosé à 1,5 %. On émulsionne les germes dans l'eau bidistillée après 24 heures de culture. Cette émulsion est ensemencée sur un milieu liquide privé de l'élément à étudier.

Au bout de 3 ou 4 jours, si le milieu noircit, on centrifuges à la vitesse de 6000 t.p.m. pour recueillir les microbes. On réensemence ce culot dans un tube de centrifugation stérile contenant le même milieu. On répète l'opération jusqu'à ce que le milieu ne noircisse plus. Ainsi la souche est épuisée et privée de cet élément (apporté par la semence).

4. Ensemencement de la culture. On ensemence le culot de la dernière centrifugation émulsionnée dans l'eau distillée, dans les fioles contenant le milieu liquide (ci-dessus). Ce milieu contient soit une certaine quantité de l'élément à doser, soit de la terre (voir dosage et étalonnage). Incuber à 36° pendant 14 jours et effectuer le dosage de la substance noire.

5. Dosage des substances noires. La mesure de noircissement s'effectue soit avec un photomètre (type à cellule photoélectrique), soit avec un comparateur optique.

Dans 100 cc de culture, on ajoute 0,5 cc de NaOH au $^1/_{10}$. Les substances noires passent en solution. On centrifuge pour éliminer les phosphates et les corps bactériens. On effectue la lecture d'absorption optique immédiatement. (On a vérifié que la densité optique est proportionnelle à la teneur en substance noire de la solution).

6. Etalonnage de la courbe. L'étalonnage de la courbe de la production des substances noires en fonction de la quantité de l'élément choisi est ainsi réalisé.

La gamme est constituée par une solution de sel (sous forme de PO_4 pour dosage de P, et de sulfates pour les autres éléments). On ajoute cette solution stérile à chaque fiole contenant 100 cc de milieu en formant une gamme de 0 jusqu'à 10 mg suivant l'échelle suivante : 0 γ, 10 γ, 50 γ, 100 γ, 250 γ, 500 γ, 750 γ, 1000 γ, 5000 γ et 10 000 γ.

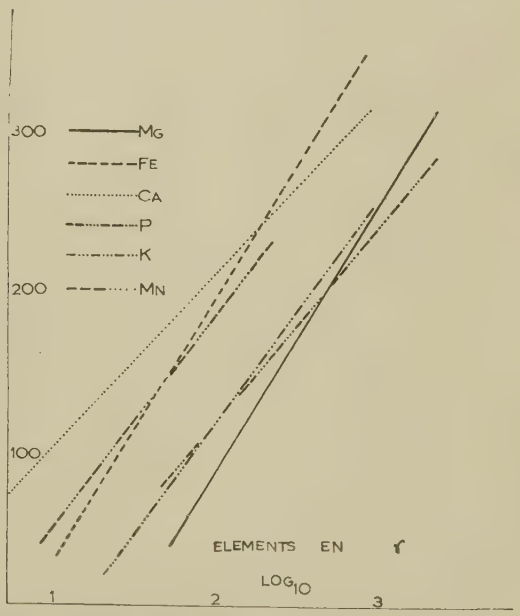
7. Détermination de la dose optima de terre à utiliser. Pour qu'un dosage d'un élément quelconque par cette méthode soit correct, il importe en premier lieu que la dose de cet élément apporté par la terre se situe dans la zone linéaire de la courbe d'étallonnage. Pour cette raison on doit faire un essai pré-

liminaire avec 0,01 g, 0,1 g et 1 g de terre dans 3 fioles contenant 100 cc de milieu privé de cet élément. On les ensemence avec une suspension *d'Azotobacter* préparée comme ci-dessus. Après 14 jours, on mesure la quantité de substance noire formée.

On choisit la dose de terre convenable pourque la quantité de substance noire produite donne une absorption comprise entre 100 et 200 division du photomètre de Meunier par example.

8. Dosage proprement dit. Utilisons maintenant 3 fioles contenant chacune 1 g de terre (par exemple). Nous faisons la mesure au bout de 14 jours en même temps que celle de la gamme étalon.

Nous trouvons pour ce cas précis (la moyenne de 3 chiffres) 190,5 divisions. Reportons nous à l'échelle étalon, notre terre contient 363 γ de P par 1 g, soit 0,363 g pour un kilogramme de terre.



En pratique le dosage s'effectue très facilement: on commence par déterminer la dose optima de terre, sans gamme étalon; dès le résultat obtenu on fait en même temps le dosage proprement dit et la gamme étalon. En moyenne, pour une terre normale, les doses de terre à utiliser sont de:

1 g pour Fe, Mn, Mg, K, P.

0,01 g pour Ca.

Il ne faut pas dépasser 1 g de terre pour 100 cc de milieu car, au delà, la coloration propre apportée par le terre peut fausser la lecture; cependant, dans ces conditions spéciales, on peut tenir compte de cette coloration de base pour établir la courbe. (Voir les graphiques en ordonnée, divisions du photomètre).

III. *Discussion.*1. Sensibilité de la méthode. Comme notre courbe a été établie avec des coordonnées semi-logarithmiques, il est évident, que la sensibilité est d'autant plus grande, que la mesure se trouve dans la zône de faibles concentrations. Nous pouvons parfaitement diviser le nombre des divisions que donne le photomètre par un chiffre quelconque pour le ramener toujours dans cette zône, puisque le produit de la densité optique par la dilution de substances noires est constant [1]).

La sensibilité et la fidélité dépendent aussi de l'expérimentateur, car une appréciation personnelle plus ou moins exacte du nombre logarithmique peut amener des différences non négligeables. (Nous nous sommes adressés à des personnes de notre laboratoire pour essayer de déterminer la variation individuelle).

Enfin on peut admettre que le photomètre peut donner une variation d'une division.

L'erreur absolue de notre méthode pour les différents éléments peut être estimée à:

P, Mg, Fe et Mn	1,0 γ
K et Ca	2,5 γ

2. Valeur pratique. L'appréciation de la fertilité du sol par l'analyse chimique est empirique. La comparaison des résultats obtenus par cette méthode et par notre méthode biologique fait ressortir qu'il y a une concordance assez acceptable pour tous les éléments dosés. Pour P notre méthode semble être plus avantageuse. En effet, pour les terres P_3 et P_{10} *) qui n'ont pas reçu de P depuis 1843, nous avons trouvé une teneur plus faible en P que celle donnée par l'analyse chimique. Nous ne pouvons par départager ces deux sortes de dosages car il faut attendre les résultats des expériences réalisées en champs avec culture sur terrains divers et pendant

*) Nos dosages ont été effectués sur des échantillons de terre envoyés de Rothamsted. Nous remercions vivement Mr le Dr. Thornton et ses collaborateurs de leur obligeance.

plusieurs années, pour apprécier les rapports entre une méthode de dosage et l'exigence des plantes.

3. Simplicité technique. Il suffit de préparer d'avance le milieu convenable pour pouvoir doser des éléments très divers par la même technique. Le milieu préconisé ne nécessite aucun produit spécial et se conserve longtemps. Le dosage peut être effectué avec un appareillage courant. L'électrophotomètre n'est pas indispensable; on peut, à la rigueur, doser les éléments par la colorimétrie visuelle (type Dubosque).

La durée de 14 jours est un peu longue, mais l'expérimentateur ne dépense que quelques heures pour effectuer un grand nombre de dosages. La durée de 14 jours est surtout occupée par la croissance des germes sans aucune intervention de l'opérateur.

Il n'y a pas d'opération délicate; on doit seulement veiller à la propreté chimique de la verrerie. On peut parfaitement confier aux personnes peu expérimentées ou à des aides techniques les analyses en séries.

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ROOT-KNOT NEMATODES

II. QUANTITATIVE RELATIONS OF THE ROOT-KNOT NEMATODE — *MELOIDOGYNE HAPLA* CHITWOOD, 1949 WITH TOMATOES, ONIONS AND LIMA BEANS

by B. G. CHITWOOD *)

The Catholic University of America, Washington, D. C.

Odd behaviors and inconsistent results in field records of experiments dealing with soil nematodes have long been known. Chitwood and Feldmesser [2]) reported on the relationships of quantity of inoculum to plant growth and resultant nematode production in the case of *Heterodera rostochiensis* on Green Mountain variety potatoes. Christie [3]) reported on some of the effects of root-knot nematodes on their hosts. The writer [1]) supplied descriptions of several root-knot nematodes.

The present experiments conducted at Beltsville, Md. were designed to test the quantitative relationships involved with one species of root-knot nematode on a few plants.

Experiment A — Tomatoes and Onions. Two hundred 8 inch tiles were sunk in the ground in a design, spaced 3 feet apart. The tiles each had a 1 inch hole in the bottom; the hole plugged with screen wire. These tiles were then filled with bank sand and 2 ounces of 5-10-5 fertilizer were added. The entire plot was randomized into blocks and columns. Half of the plots were allocated for the planting of tomatoes, and half for the planting of onions.

Inoculum was grown on tomato plants, the roots were rinsed, chopped up finely and the quantity of egg masses per gram of root determined by alliquote weight samples. Inoculum was weighed out equivalent to 12.5, 38 and 300 egg masses. Individual egg masses were picked out for single egg mass inoculations. The tiles were then

*) Formerly Division of Nematology, Bureau of Plant Industry, Soils and Agric. Eng.

inoculated with 0, 1, 12.5, 38 and 300 egg masses according to design. Thereafter half of the plots were planted with small marglobe tomato plants and half with yellow globe onion sets. Planting was on May 14th, 1948. Records were taken on Aug. 6 and Sept. 6, 1948.

TABLE I

Relationships of *Meloidogyne hapla* to tomato plants as of July 6, 1948

Inoculum in egg mass.	Mean Top Wt in grams	Mean Root Wt in grams	Visual root gall index	Mean ♀/Pl	Mean EM/Pl
0	23.8 ± 4.7	12.1 ± 2.4	0.0 ± 0.0		
1	38.0 ± 6.1	18.5 ± 4.2	0.9 ± 0.3	5.8 ± 3.0	2.6 ± 1.6
12.5	31.5 ± 9.8	12.6 ± 1.1	1.6 ± 0.3	62.7 ± 28.4	18.8 ± 6.6
38	14.7 ± 2.7	7.4 ± 1.1	2.8 ± 0.5	137.9 ± 20.9	45.5 ± 13.1
300	21.5 ± 5.4	13.1 ± 3.1	4.0 ± 0.4	333.8 ± 43	166.8 ± 28.9

TABLE II

Relationships of *Meloidogyne hapla* to tomato plants

Harvest Inoculum in egg masses	Record Aug. 6, 1948, Mean Plant Growth Index	Mean Top Weight in grams	Mean Root Weight in grams	Mean Yield in grams	Visual Root Gall Index	Mean ♀ specimens per plant	Mean egg masses per plant
0	4.9 ± 0.8	47.5 ± 18.8	13.9	93.7 ± 50.5	0 ± 0		
1	4.8 ± 0.5	22.5 ± 4.9	13.4	9.1 ± 7.2	1.4 ± 0.3	173 ± 18	38 ± 8
12.5	4.5 ± 0.8	23.9 ± 6.3	11.5	25.1 ± 8.5	2.6 ± 0.2	706 ± 161	254 ± 70
38	4.0 + 0.5	23.9 ± 5.5	14.6	14.4 ± 4.0	3.8 ± 0.4	741 ± 109	476 ± 61
300	3.2 ± 0.1	14.6 ± 2.2	12.0	3.7 ± 2.8	4.2 ± 0.1	1115 ± 256	811 ± 142

Results. Tabular records of the records from the tomato plants are given in tables I and II. From table I it will be easy to see that the visual root gall index increased during the first growth period with quantity of inoculum. The numbers of females and egg masses per plant increased in a similar manner but the increase was not in proportion to the increased dose of inoculum. Actually we see a progressive decrease in the rate of reproduction as based upon counts of either females or egg masses. There was a net decrease in the resulting number of egg masses for the maximum quantity of inoculum.

During the second growth period, as indicated by table II the differences in plant growth as evidenced by top growth and yielp

TABLE III

Rates of Reproduction (RX) of *Meloidogyne hapla* on Marglobe tomatoes
(*a*) Expressed in females

Inoculum in egg masses	Period of time 5/14–7/6/48		Period of time 7/6–8/6/48	
	RX♀	RXEM	RX♀	RXEM
0	0	0	0	0
1	5.8	2.6	30	15
12.5	5.0	1.5	11	13
38	3.6	1.2	5.4	11
300	1.1	0.6	3.3	4.9

were quite pronounced but the differences in numbers of females and egg masses were much less marked. Roughly speaking the ratio of yield in egg masses to inoculum in egg masses (RXEM) is reduced with increase of inoculum but the final result depends upon the amount of plant root present at any given time.

The records taken relative to yellow globe onions *) are not given since they were so outstanding. Suffice it to say that as of the first date, July 6, 1948, a mean of 28 female nematodes per tile reached adulthood and 0.6 egg mass per tile in the 50 tiles (with all grades of inoculum allocated to the test). As of the second date, Aug. 6, 1948, no egg masses were found though a few galls were present on the roots. Inside the galls hard sand-like particles were commonly. Some onions appeared to have been killed as a result of the first invasion with the heaviest inoculum but those that survived grew well with no evidence of nematode damage.

Experiment B — Lima Beans †). Three varieties of lima beans were grown, 12 pots of each variety for each of 3 levels of inoculum, 0, 30, and 300 egg masses. The treatments were randomized. After 60 days the tops, roots and pods were weighed, the root-gall index taken and the roots were chopped finely, aliquote weights taken and the nematodes per gram of root were determined. (Table IV). One of the most interesting bits of information provided by this test is that the greater the amount of inoculum the fewer mature nematodes produced in proportion to inoculum. The initial effect of a moderate

*) These statements based on observations by Kenneth Demaree.

†) This experiment was conducted in cooperation with R. E. Wester and H. Jorgensen in a greenhouse at Beltsville, Md.

TABLE IV

Relation of soil inoculum, root-gall index, nematodes per gram of root and plant response in 3 varieties of Lima beans. All figures are mean.

Variety	Inoculum in egg masses	Root gall Index	Weights in Grams			Pod No.	Nematode Females		
			Top	Root	Pod		Per g Root	Per plant	RX♀
Henderson	0	0.0	25	7.6	26	7	0	0	
	30	2.5	36	15.3	25	10	199	3045	101
	300	4.6	34	13.8	22	10	345	4744	16
Asgrow Fordhook	0	0.1	64	15.6	28	4	0	0	
	30	2.3	76	20.6	36	5	92	1895	63
	300	4.1	56	25.2	21	3	249	6225	21
Fordhook 242	0	0.0	64	20.1	42	6	0	0	
	30	2.4	79	25.8	40	9	75	1935	64
	300	3.6	68	27.1	26	4	222	5939	20

quantity of nematodes was to kill some roots, stimulating the remainder to proliferate. Nematodes per gram of root paralleled the root gall index but nematodes per plant did not. We have here a complicated interaction of quantity of incoulum, quantity of root at date of invasion, and response of the plant variety to attack by the nematode. If the new roots are not killed the stimulation may be reflected in top growth, pod number and yield.

Though Fordhook 242 had a significantly lower root gall index than Henderson, this was due to dilution by virtue of the greater root mass. Such a condition had best be termed tolerance.

Conclusions. The quantity of *Meloidogyne hapla* produced on plants depends on the amount of inoculum, the amount of plant present at the moment of root invasion, the plant family, genus, species and variety. Temperature is also a governing factor but this item was not tested in the present experiments. The effect of the nematodes on the host is likewise a complicated interaction.

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MOLYBDENUM NUTRITION OF CROP PLANTS

I. THE INFLUENCE OF PHOSPHATE AND SULFATE ON THE ABSORPTION OF MOLYBDENUM FROM SOILS AND SOLUTION CULTURES

by P. R. STOUT, W. R. MEAGHER, G. A. PEARSON and C. M. JOHNSON

Division of Plant Nutrition, University of California Agricultural Experiment Station, Berkeley, California U.S.A.

The object of this paper is to report further investigations of the role of phosphate and sulfate levels of soils and culture solutions as they affect molybdenum absorption by plants. It was reported earlier [28]) that tomato plants absorbed and translocated sub-microgram amounts of radioactively tagged molybdate from single salt solutions of phosphate, chloride, nitrate, and sulfate. The greatest translocations of molybdate took place when present with phosphate salts and the least in the presence of sulfates, the difference being very large, in the order of 10 fold. As compared to the marked differential effects of phosphates and sulfate the influence of different cations on molybdate absorption was negligible. The role of sulfate ions in suppressing molybdate absorption and translocation may be explained as a direct competition between two divalent anions of the same size. However, no simple chemical explanation can be offered for enhanced accumulation of molybdate in the shoots of plants with increasing phosphate levels in the culture solution. Since in all instances roots absorbed molybdate, the amounts translocated must be related to physiological processes governing the release of root absorbed molybdate to the plant's conduction system. Inorganic phosphate levels must assume a dominate role in effecting the release of molybdate from root cells.

The present studies cover a wide variety of experimental arrangements designed to study factors influencing the molybdenum nutri-

tion of plants. Some of the investigations with soils were in progress prior to the solution culture experiments referred to [28]), and others have been initiated later. In all instances corroborative evidence of interrelations between phosphates, sulfate and molybdate have been found. Moreover, the effects were highly consistent under such very different experimental circumstances as short time absorption from single salt solutions and long term experiments involving pot cultures of plants grown to maturity on soil. Because of the observed consistency, generalizations of the phenomena are permitted which may be of use in field management wherein molybdenum absorption by plants presents practical agricultural problems.

The consequences of molybdenum absorption by plants may be divided into two distinctively separate catagories. One deals with the molybdenum nutrition of plants *per se*, and the other with adverse effects in animal nutrition arising from ingestion of excessive amounts of molybdenum in feeds.

For our purpose, we have correspondingly differentiated between soils of high and low molybdenum supplying power. The division is arbitrary, and is based on the molybdenum content of plants grown upon them without regard to species. Our present experiments involved tomatoes, peas, and subterranean clover. Analyses of tomato and pea plants grown under comparable conditions show somewhat lower concentrations of molybdenum in the tomato plant. It has been suggested [7]) that legumes as a class have greater power of accumulating molybdates than non-legumes. However, this generalization has not been borne out in other unreported experiments we have conducted. Having analyzed 35 species of crop plants grown on soils at both high and low soil molybdenum levels under otherwise similar cultural conditions, we can now only state that the powers of individual plant species to accumulate molybdenum are greatly different. For example on the high molybdenum soil the greatest accumulation was shown for broccoli, but upon low molybdenum soils the molybdenum absorbed by broccoli was exceeded by many other species. In characterizing soils with respect to their molybdenum supplying power, we have chosen to rate them according to the molybdenum concentrations accumulated by growing plants. Analyses of molybdenum deficient plants made in our laboratory have ranged from 0.25 to 0.03 p.p.m. dry weight. Walker [30]) has reported 0.11 p.p.m. and less for molybdenum deficient tomato plants grown on a

serpentine soil. Considerable variation has been found for different species, with further modifications associated with cultural conditions. From the point of view of molybdenum as an essential element for plant growth, we have tentatively set the figure of 0.5 p.p.m. molybdenum in the dry plant material as indicative of an adequate molybdenum supplying power of soils or other nutrient substrates. On the other hand soils providing in the order of 10 p.p.m. of the dry weight of plants have become known as high molybdenum soils. Concentrations of molybdenum in pasture plants of 10 p.p.m. and greater has been associated with the teart disease or molybdenosis of cattle and sheep [7], [9], [16], [23]. Intermediate concentrations lying between approximately 0.5 and 5 p.p.m. molybdenum at present are not recognized as of consequence in either plant or animal nutrition.

As will be shown, the influence of phosphates in enhancing molybdate absorption and of sulfate in repressing it, is of such magnitude that the ability of soils to supply molybdenum to plants must be judged in terms other than concentrations of soil molybdenum alone.

With respect to significant levels of soil molybdenum involved in these studies, it may be pointed out that certain low molybdenum soils have been effectively converted to ones of very high molybdenum power through surface application of one part of soluble molybdenum per million parts of soil. Also, depending upon differential soil treatments with sulfate and phosphate, concentrations of molybdenum in plants have been caused to vary in the order of 10 fold.

At the time these studies were begun, interest in the role of molybdenum in plant nutrition was rapidly increasing, principally because of the remarkable field results obtained by Australian workers who were successful in increasing the yields of legumes through molybdenum applications in the field. Interpretations of the field responses to molybdenum in Australia as reviewed by Anderson [1], Anderson and Thomas [4], and Anderson and Oertel [3] were that subterranean clover showed a secondary response to molybdenum applications through the symbiotic nitrogen fixing organisms. It did not seem that the soils were in reality molybdenum deficient for plants *per se*, since no molybdenum response was obtained when their plants were fertilized with nitrates. However, the results of Davies [11] in New Zealand who associated the whiptail disease

of cauliflower and cabbage with molybdenum deficient soils, made it appear that the New Zealand soils either had a lower molybdenum supplying power than the soils in Australia, or that cabbage and cauliflower were plants having an exceptionally high molybdenum requirement.

There were several reasons for expending some effort toward finding soils of low molybdenum supplying power in California. First, it seemed reasonable that if the Australian soils referred to had reached a molybdenum level low enough to effectively, though indirectly, limit the growth of legumes, an intensification of the weathering factors giving rise to low molybdenum levels should reduce the molybdenum supplying power sufficiently to give rise to molybdenum deficiency for any plant specie. Second, an available supply of soil material capable of producing molybdenum deficiency in plants would permit controlled experimentation with enough different species to enable us to secure data with respect to the quantitative levels of soil molybdenum of significance in plant nutrition. Third, from the point of view of soil chemistry, an explanation of chemical weathering in relation to types of parent material involved in molybdenum supplies for plants, would represent a contribution to the general field of soil chemistry; and fourth, experience gained with growing plants on low molybdenum soils would of course be of value in extending knowledge of plant and soil factors involved in the complete mineral nutrition of plants.

In 1935, van Niel [29]) had shown that a slightly alkaline sandy soil collected at Carmel, California, was too low in molybdenum to successfully grow *Azotobacter* when dependent on the atmosphere for nitrogen. In 1939, since it was known that molybdenum was required for the growth of tomatoes [6]), one of the writers (P. R. S.) and D. I. Arnon reasoned on the basis of van Niel's reports [29]) that soils might exist which were sufficiently low in molybdenum to show deficiency symptoms in crop plants. Being familiar with foliar symptoms of molybdenum deficiencies produced from water cultures, field examinations in the California coastal tomato growing regions were made for suggestions of similar disorders in field grown crops. However, no visually recognizable molybdenum deficiency symptoms were found, at least as they were known from molybdenum deficient plants grown in solution cultures.

A distinctly opposite problem, presented by excessive amounts of

molybdenum absorption by plants, exists in some parts of California; namely, molybdenosis of cattle. Factors contributing to the disease in California have been studied by Britton and Goss [9]) and Barshad [7]). It appears to be similar to the teart disease first recognized in England as reported by Lewis [16]). Molybdenosis, or the teart disease of ruminants becomes evident when their food contains in the order of 10 parts per million or more of molybdenum. Because of van Niel's work with *Azotobacter* on a low molybdenum soil [29]) and that of Britton and Goss with molybdenosis on high molybdenum soils [9]), it was obvious that molybdenum levels of California soils varied enormously.

In seeking soils of low molybdenum supplying power, it was thought that better chances of success would lie in regions where natural weathering processes were associated with freely draining surface and ground waters. In view of the Australian and New Zealand experiences associated with low molybdenum soils [1]), [2]), [3]), [4]), [11]), [13]), [19]); we were particularly interested in acid leached soils, sufficiently weathered to give rise to phosphate deficiency, but which still did not give good growth of legumes after being fertilized with N, P, K, S, Ca and Mg. Through the cooperation of the University of California Extension Service *), we were able to secure samples of acid soils which as a result of field fertilizer trials appeared to meet the above requirements.

The selected soils, described below did not produce molybdenum deficient plants, even though techniques [27]) found successful for controlled micronutrient element experiments with water cultures were applied to the soil cultures in pot tests. However, in the progress of the work it was noted that fertilization with phosphate apparently enhanced molybdenum absorption by plants. It was thought that this phenomenon might be of sufficient importance in the molybdenum nutrition of plants to deserve special study.

Molybdenum absorption by plants from soils selected as potentially low in molybdenum supply. Description of soils: Soils *a* and *b* were taken from a farm located near the coast in Marin County. The lands have not yet been mapped by the Soil Conservation Service, but the

*) We gratefully acknowledge the active cooperation of Dr. W. E. Martin, Extension Specialist in Soils.

soils are residual clay loams formed on an old marine terrace. The bedrock is of considerable depth. Field fertilizer trials showed only a slight response to phosphorus on legumes and no appreciable response by grasses or other vegetation to nitrogen, potassium, or sulfur alone or in combination with phosphorus. Greenhouse pot tests had shown a slight depressing effect from the addition of potassium and an increase in yield of leaf lettuce when lime was added.

Soils *c* and *d* are the surface and sub-soil respectively, taken from an apple orchard west of Petaluma, Sonoma County, California. It is classified as Gold Ridge fine sandy loam, a residual soil of low fertility formed from slightly consolidated sandstone, in a high rainfall zone. Soil *C* was taken from the surface 8 inches, and Soil *D* from the 20–24 inch depth were most of the apple roots grew. Trees growing on this soil were badly affected with apple die-back for which no cause has yet been found. Vetch grows well in this area provided phosphorus is added, but sweet clover requires the further addition of lime for success. Indications of response of apples to boron and zinc have been noted although neither of these elements alone or together have controlled the disease of apples. Also the symptoms of the disease shown did not seem to be completely compatible with those regularly shown by zinc and boron deficiencies.

Soil *e* was of particular interest because it had shown plant responses to manure similar to those reported for low molybdenum Australian soils. The sample represented the first 8 inches and was from a site mapped as Empire fine sandy loam, located in Humboldt County in Northwest California. This soil was brought to our attention because dififculty had been experienced with growing peas. All of the field had been treated with superphosphate, and the peas were inoculated. Only two sections of the field produced a crop. In one section where sodium nitrate had been applied at the rate of 40 pounds per acre, the crop was fair, but no nodules were observed. In another small spot in the field, growth was good and good nodulation was observed. The latter spot was one where "foreign" sheep manure had been piled prior to spreading throughout the field. It was believed that the nitrogen from the remaining sheep manure could not account for the response observed. Secondary effects of trace elements of benefit to the legume bacteria were suspected.

Method of greenhouse cropping. Because of Anderson's [1] [2] [4] experiences that low molybdenum levels in soils

were evidenced by failure of legumes to fix nitrogen, a legume was chosen as a test plant, on the assumption that it would be more likely to reflect a low soil molybdenum level through the higher molybdenum requirement of symbiotic nitrogen fixing micro organisms. Subterranean clover was specifically selected because of its tolerance to acid soils. Also in accord with Anderson's report that molybdenum applications of $^1/_4$ to 1 ounce of MoO_3 per acre were as effective as 4 pounds per acre, we arbitrarily decided upon molybdenum treatments of 3 mg Na_2MoO_4 per pot containing 2 kilograms of moist soil. Four millimols of $Ca(H_2PO_4)_2$ were also applied. On an area basis, the respective treatments corresponded to 1.0 pounds of molybdenum and 405 pounds P_2O_5 per acre. Clover plants were grown on the five different soils for 12 weeks. Yields and molybdenum concentrations are shown in Table I.

TABLE I

Yield and Molybdenum Content of Subterranean Clover Grown in Soils Fertilized with Molybdenum and Phosphates

	First Crop									
	Soil A		Soil B		Soil C		Soil D		Soil E	
Treatment [1])	Yield g [2])	Mo p.p.m.	Yield g [2])	Mo p.p.m.	Yield g [2])	Mo p.p.m.	Yield g [2])	Mo p.p.m.	Yield g [2])	Mo p.p.m.
None	2.2	0.18	4.1	<.21	2.2	0.8	0.3	4.3	4.1	0.21
,,	1.9	0.20	4.5	<.10	2.2	0.4	0.4	2.4	3.7	0.28
Mo	2.2	4.3	3.1	11.8	2.4	91.2	0.3	82.0	4.7	6.4
,,	1.8	8.0	5.1	10.8	3.4	44.6	0.5	54.2	4.9	7.9
PO_4	5.1	0.9	7.2	0.4	2.8	0.8	0.1 *)	7.0	6.1	0.27
,,	6.2	1.8	7.4	0.7	3.4	0.6	0.1 *)	3.3	8.6	0.28
Mo+PO_4	5.0	79.3	8.1	27.2	3.5	109	0.1 *)	58.3	6.2	123.5
,,	5.3	61.4	7.5	79.0	2.9	153	0.04	68.5	8.2	69.1
pH, 1:2 susp.	5.5		5.6		4.8		5.1		4.9	

[1]) Applied salts were added dissolved in 400 cc of water. The solution was poured quickly over the loose soil mass in order to distribute it throughout the entire sample.

Treatments to 2 kilograms of undried soil as collected from the field

1. 400 ml of distilled water only.
2. 400 ml water containing 3 mg of Na_2MoO_4 (1 pound Mo per acre).
3. 400 ml water containing 4 m. mols $Ca(H_2PO_4)_2$ (405 pounds P_2O_5 per acre).
4. 400 ml water containing 4 m. mols $Ca(H_2PO_4)_2$ plus 3 mg Na_2MoO_4.

[2]) Yields are reported as total dry weight per pot of the above ground part of the plant.

*) Plants were dead at time of harvest.

In the pot cultures distinct phosphate responses were obtained with the Marin County soils A and B, and with the Humboldt county

soil E. Of the soils from Sonoma County, the surface soil (Soil C) supported subterranean clover plants but showed no significant response to phosphates. The subsoil (Soil D) however, barely supported only a scanty growth which was materially depressed by application of phosphate.

Although at no time during the cropping period were any visual effects observed that could be ascribed to molybdenum deficiency, subsequent analyses of the plants were encouraging in that the untreated soils had produced plants having a lower molybdenum content than we had previously found. For example, in Table I molybdenum concentrations in clover grown on soils A. B, and E were found to be .18, .20, < .12, < .10, .21 and .25 p.p.m. Ter Meulen[17]), Barshad[7]) and Robinson and Edgington[23]) who have reported molybdenum concentrations in various plant materials ranging from .3 p.p.m. upward to 120 p.p.m.

Our values for molybdenum here obtained appeared more nearly comparable with those of Anderson and Oertel[3]) who reported that healthy lucerne and *Phalaris tuberosa* grown on burned over land contained respectively 0.7 and 0.3 p.p.m. molybdenum whereas poorly developed plants on an adjoining area contained less than 0.1 p.p.m. This difference in the molybdenum content of plants on the two areas provided them with one of their earlier clues that the effective agent contributing to better plant growth in burned over areas was molybdenum from plant ash.

Influence of phosphate fertilization of certain acid soils in increasing molybdenum absorption by plants. The principal point of interest in the data of Table I is that for a given level of soil molybdenum, application of phosphate at the rate of 400 pounds P_2O_5 per acre caused an enormous increase in the amount of molybdenum taken up by the plants. Not only was the concentration of molybdenum in the phosphate treated plants increased, but even greater increases were obtained in the total amount of molybdenum absorbed since phosphate fertilization also increased the yields.

For example, in Table I, plants grown on Soils A and E, had 8.0, 4.3, 6.4, and 7.9 p.p.m. molybdenum when only molybdenum was applied but 79.3, 61.4, 123.5, and 69.1 p.p.m. when fertilized with phosphate. The marked increase in molybdenum uptake associated with the higher phosphate level cannot be ascribed to molybdenum

contamination in the phosphate fertilizer since treatment 3, phosphate alone, showed but 0.9, 1.8, 0.27, and 0.28 p.p.m. molybdenum in plants grown on the same soils.

Anderson and Oertel[3]) indicated no particular influence of phosphate fertilization on the amount of molybdenum extracted by plants. Conditions imposed by these experiments, however, unmistakably demonstrate that phosphate levels in soils are important variables related to the molybdenum accumulation by plants. This interrelation with phosphate was very prominent with soils A, B, and E. The same trend though less pronounced, is shown for soil C. Similar comparisons cannot be made for Soil D because of crop failure on the phosphate treated pots.

It was assumed that calcium did not contribute to variations in the plant responses as shown by molybdenum absorption, since the calcium added with the $Ca(H_2PO_4)_2$ was shown by soil analyses to amount to only 2% or less than the soluble and exchangeable calcium already present in the soil. Other soil and culture solution experiments discussed later have adequately corroborated this assumption.

In order to show the effect of phosphate on the total molybdenum absorbed, data from plants grown on soils A, B, and E have been compiled in Table II. Columns 3 and 4 show respectively the relative increase in *molybdenum concentration*, and relative increase in *total molybdenum removed* by plants resulting from phosphate fertilization. Column 5 shows the percent recovery of 1 pound per acre of added molybdenum, with and without phosphate fertilization.

Ratios in column 3 showing increased concentration of molybdenum as a result of phosphate fertilization are in some instances very large. The increases are particularly evident when phosphate and molybdenum were added in combination, the molybdenum concentration being increased ten to thirty fold over that without added phosphate. The phosphate effect is thus seen to be operative at two widely different levels of soil molybdenum.

Increased molybdenum uptake resulting from phosphate fertilization is even greater when considered from the point of view of total amounts of molybdenum removed by plants (Column 4) since the molybdenum concentration was increased, even though higher yields were obtained.

Percent recovery of added molybdenum also illustrates the same

TABLE II

Influence of Phosphate Treatments on the Molybdenum Uptake by Tops of Subterranean Clover

	1 P.P.M. Mo			2 Micrograms Mo Removed by Tops			3 Factor showing Increased Conc. of Mo due to PO_4 fertilization			4 Factor showing Increased Total Removal of Mo. Due to PO_4 fertilization			5 Percent Recovery of Added Mo		
Soil	A	B	E	A	B	E	A	B	E	A	B	E	A	B	E
Treatment															
1. Check	0.18	< 0.21	0.21	0.4	< 0.86	0.90									
	0.20	< 0.10	0.28	0.4	< 0.45	1.00	—	—	—	—	—	—	—	—	—
2. Plus molybdenum	4.3	11.8	6.4	9.5	36.6	30.0	—	—	—	—	—	—	0.62	2.5	2.0
	8.0	10.8	7.9	14.2	55.0	39.0	—	—	—	—	—	—	0.95	3.7	2.6
3. Phosphate only	0.9	0.4	0.27	4.6	2.9	1.6	4.7	>2.7	1.1	11.5	> 4.4	1.7	—	—	—
	1.8	0.7	0.28	11.2	5.2	2.4	9.5	>4.7	1.1	28.0	> 7.9	2.5	—	—	—
4. Molybdenum plus phosphate	79.3	27.2	123.5	396	220	760	12.8	2.4	17.3	33.5	4.8	22.0	26.8	14.9	53.6
	61.4	79.0	69.1	325	590	580	9.9	7.0	9.7	27.5	12.9	16.8	21.8	40.4	39.8

The letters A, B, E, at the head of each column refer to plants grown in soils A, B, and E respectively.

Column 1. Concentration of molybdenum, p.p.m. dry weight, found in the tops.

,, 2. Micrograms of molybdenum removed by tops of plants grown on a single pot.

,, 3. Factor showing the increase in molybdenum concentration due to treatment with phosphate — obtained by calculation, using as a reference the average concentration of molybdenum in plants not treated with phosphate, but which received the same molybdenum treatment.

,, 4. Increased total removal factor due to treatment with phosphate, obtained by calculation, using as reference the average total amount of molybdenum removed by the tops of plants not treated with phosphate, but which received the same molybdenum treatment.

effect, as may best be seen in Column 5, table II. Without supplemental phosphate (treatment 2) the lowest recovery of added molybdenum was 0.62% and the highest was 3.7%. With the addition of 400 pounds P_2O_5 per acre, however, the percent recovery was greatly increased, the lowest percent recovery being 14.9% and the highest 53.6%.

Although somewhat outside the scope of the principal subject of this paper, at least two quantitative aspects of the molybdenum accumulating power of clover plants, as revealed by these data, deserve comment in connection with excessive concentrations of molybdenum in leguminous cattle feeds. As earlier mentioned, workers concerned with molybdenosis have regarded molybdenum concentrations of 10 parts per million as a borderline figure above which the frequency and severity of the disease is likely to be increased.

It is seen from the results of Table I that large increases in the molybdenum content of plants may be induced solely through the application of phosphate fertilizers even though soil molybdenum is the same. It is therefore conceivable that the molybdenum content of hay could be increased from a safe to an unsafe molybdenum concentration through the indirect means of fertilization with materials other than molybdenum itself. For example, in Table I compare analyses of plants from the individual pots of treatment 2 (molybdenum only) with those of treatment 4 (molybdenum plus phosphate). As a result of adding phosphate to Soil A, the molybdenum contents of plants were raised from 4.3 and 8.0 p.p.m. to 79.3 and 61.4 p.p.m. Similarly for Soil B the molybdenum content of clover was raised from 11.8 and 10.7 p.p.m. to 27.2 and 79 p.p.m. Also, plants grown on Soil E were raised from 6.4 and 7.9 p.p.m. to 123 and 69 p.p.m. It may be noted that when molybdenum alone was added to Soil D the molybdenum concentration was raised from 4.3 and 2.4 p.p.m. to 82.0 and 54.2 p.p.m. (cf. Soil D, treatments 1 and 2 Table I) even without phosphate fertilization.

A second point of interest with regard to high molybdenum soils is that the enhanced molybdenum uptake induced by phosphates effects rapid removal of soil molybdenum. This may be seen in the data of Table II which shows that the addition of phosphate resulted in the recovery of a substantial portion of added molybdenum by a single cropping. The average percent recovery of molybdenum in the first crop from soils A, B, and E, treatment 2 (molybdenum alone) is

2.06%. An average percent recovery of 32.9 however, was obtained from the same three soils of treatment 4 (molybdenum plus phosphate).

Molybdenum absorption by garden peas following cropping with subterranean clover. After harvesting the subterranean clover, the same pots were replanted to garden peas without further fertilization. The second crop was harvested and followed by a third crop, also of garden peas. The pea plants from each pot and for both harvests were analyzed for molybdenum. Molybdenum concentrations of the second crop and third crop are given respectively in tables III and III*a*.

TABLE III

Yield and Molybdenum Content of Garden Peas Grown in Soils Fertilized with Molybdenum and Phosphates

	Second Crop									
	Soil A		Soil B		Soil C		Soil D		Soil E	
Treatments *)	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.
1. None	2.69	1.6	2.49	2.1	2.30	2.1	1.76	2.6	2.06	2.5
	2.48	1.7	2.43	2.0	1.97	2.3	1.48	4.1	2.04	2.3
2. Mo	2.22	3.5	2.69	3.3	2.42	4.5	1.94	13.5	2.30	4.5
	2.83	3.6	2.50	3.3	2.45	4.8	0.99	22.5	1.67	3.8
3. PO_4	3.10	1.4	3.52	0.8	3.05	1.9	1.87	2.8	2.25	1.4
	3.69	1.0	2.97	0.9	2.40	1.4	1.81	1.9	2.40	2.3
4. PO_4+ Mo	3.61	2.4	2.89	2.3	2.47	4.8	1.65	21.2	2.23	4.9
	2.96	2.5	3.26	2.9	3.11	3.5	1.30	27.8	2.08	4.8

*) Treatments were made before the first crop of subterranean clover. For method of treatment, refer to Table I. Also note, data for each replicated culture occupy the same positions as data in Tables I and IV.

The marked effects of phosphate fertilization shown so strikingly in the first crop disappeared in both the second and third crops. Reasons for such behavior are not clear but may have arisen from a number of different factors having to do with the chemistry of the soil system or to physiological peculiarities of the different plant species used.

For some soils, trends shown in the first crop appeared reversed in the second. For example, in the first crop (Table I) clover plants grown on soil A accumulated 4.3 and 8.0 p.p.m. molybdenum when treated with molybdenum alone, and 79.3 and 61.4 p.p.m. with added phosphate. Molybdenum concentrations for the respective

treatments in the second crop (Table III) were 3.5 and 3.6 p.p.m. for molybdenum alone, and 2.4 and 2.5 p.p.m. when treated with phosphate. In the third crop (table III*a*) molybdenum alone gave 3.6 and 2.9 p.p.m. and molybdenum plus phosphate 5.0 and 3.1 p.p.m.

After the first crop, further evidences of interrelations between phosphate fertilization and molybdenum accumulation by plants were found to be variable among the several soils. With soil D the effect of phosphate in increasing molybdenum absorption remained throughout all three crops, but with the other soils no consistent trend could be shown after the first crop. However, results of the

TABLE III*a*

ield and Molybdenum Content of Garden Peas Grown in Soils Fertilized with Molybdenum and Phosphates

	Third Crop, Garden Peas									
	Soil A		Soil B		Soil C		Soil D		Soil E	
reatment *)	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.	Yield g	Mo p.p.m.
None	2.3	0.52	3.8	1.3	3.4	1.2	2.0	2.6	3.4	1.7
	3.3	1.5	4.0	1.5	1.45	2.6	1.7	2.8	3.8	2.0
+ Mo	3.1	3.6	3.95	3.7	3.3	7.6	2.04	9.4	2.92	3.3
	4.4	2.9	4.0	3.2	4.6	6.2	2.0	12.5	3.4	3.6
+ PO_4	4.8	1.4	4.7	0.9	5.7	1.1	0.6	1.8	4.35	1.56
	4.4	1.0	4.3	1.1	3.3	1.8	1.1	1.4	3.36	1.8
+ PO_4 + Mo	4.6	5.0	5.4	2.8	4.0	12.9	3.9	18.8	3.60	3.4
	4.5	3.1	4.6	2.5	4.9	4.7	1.24	27.5	3.42	3.4

*) Treatments were made before the first crop of subterranean clover. For methods of treatment, refer to Table I, also note, data for each replicated culture occupy the same positions as data in Tables I and III.

first crop left no doubt as to an association between the phosphate levels of soils and their influence on molybdenum absorption by plants. Reasons for such effects had to be looked for with simpler systems than the complex soil medium.

An early explanation was that phosphates added to the soil might have resulted in release of molybdate from the anion adsorption complex, thus making soil molybdenum available to plants. Such exchange systems had been postulated by Stephens and Oertel [25] to explain plant responses to molybdenum when low molybdenum acid soils were limed. Later with similar soils, Anderson and Oertel [3] in a series of ingeniously designed experiments,

were able to conclusively demonstrate that increased nitrogen fixation by clovers was due to rendering molybdenum available when the soils were made less acid through liming.

Fixation and exchange of molybdate ions by soil clays. Since hydroxyl ions are effective in replacing adsorbed phosphate ions [12] [26], there was reason to believe that if molybdates were in fact adsorbed by soil colloidal materials they should be similarly replaceable by hydroxyl ions. So far, however, the only anions recognized by soil chemists as being strongly fixed in exchange processes with soil colloidal materials are phosphate, arsenate, fluoride and hydroxyl ions. The exchange properties of molybdate should approach those of sulfate since the two ions are of the same size and charge. Sulfate, however, is not usually regarded as being fixed by soil colloidal materials, although Reitemeier [22] has indicated that important amounts were fixed in adsorbed form by a moderately acid Palouse soil.

Adsorption of molybdate ions by halloysite by anion exchange. In order to check the adsorption of molybdate by a pure clay material of known structure, adsorption experiments were conducted with hydrated halloysite. Hydrated halloysite is an ideal clay mineral for studying anion exchange reactions since its crystallographic structure is characterized by an entire sheet of hydroxyl ions at one side of each neutral kaolin like packet. Moreover, it is known [26] that its exposed lattice hydroxyl ions readily exchange for phosphate ions. It was expected that if molybdates were capable of participating in similar exchange reactions, they should also be adsorbed by this clay material.

An experimental column of hydrated halloysite was prepared as follows: One hundred grams of hitherto undisturbed lumps of hydrated halloysite were crushed in a mortar and passed through a 1 mm sieve. The screened material was suspended in distilled water and the fine material was decanted several times. A glass cylinder 2.7 cm in diameter was then filled with the halloysite to a height of 11 cm. The resulting column permitted a relatively high percolation rate of $2^1/_2$ ml per minute with a total head of 15 cm of water.

In order to remove soluble molybdenum, the column was leached with 0.1 N Na_2CO_3. Successive 50 ml portions of the leachate were

analyzed for molybdenum. The first increment of 50 ml passing through the column removed one microgram of molybdenum. No detectable amount of molybdenum appeared in the fourth leaching. A total of 1.8 micrograms molybdenum was removed by the four leachings.

Following the sodium carbonate wash, the column was leached with .02 N HCl until all alkali disappeared. It was further washed with distilled water until free of chlorides.

Fifty ml of water containing 300 micrograms molybdenum as Na_2MoO_4 were then passed through the column followed by leaching with distilled water. Four successive 50 ml aliquots of the leachate were analyzed. No molybdenum could be detected in 200 ml of the leachate showing that the molybdate had been completey fixed.

In order to determine the position of the molybdenum, within the column the halloysite block was removed and cut into seven 1.5 cm segments. Molybdenum in the separate segments was displaced from the halloysite by heating each segment with 50 ml of 0.5 N Na_2CO_3. After warming for 2 hours on the steam bath, the solutions were decanted and analyzed. The first five segments beginning from the uppermost, released 239, 0.08, 1.9, 1.1, and 1.7 micrograms of molybdenum respectively. The last two segments were not analyzed.

This experiment showed molybdates to be strongly fixed by halloysite. It also showed that much of the fixed molybdenum was released by moderately alkaline solutions presumably by an anion exchange process.

Because of the completeness of removal of molybdates by the halloysite from a water solution, it was thought that similar columns might be effectively used as a means of separating trace amounts of molybdenum from some of our stock salt solutions used for plant cultures and fertilizers. However, substantial quantities of molybdates were found to pass through the column with the percolate when a molar solution of $MgSO_4$ containing molybdates was substituted for water. It was assumed, pending further investigation, that in molar salt solutions of sulfates molybdates had been less effectively adsorbed by halloysite because of competition of the sulfates for anion exchange positions.

The chemistry of molybdate adsorption and exchange in soil materials is the subject of more extended investigations currently in progress in our laboratory. Goldberg[15]) with more refined

chemical procedures has examined molybdenum fixation by soils and soil minerals over a wide range of pH values and finds that the degree of fixation continues to increase with acidity until pH values as low as 2.2 are reached, and that all molybdenum fixed by exchange can be released with alkaline solutions of pH 9. He has also shown that soluble phosphates can exchange for adsorbed molybdates.

Thus enhanced molybdenum absorption by plants from phosphate fertilized soils could be explained in part by replacement of soil molybdate from the anion exchange complex by phosphates.

Absorption of molybdenum from culture solutions in short time absorption experiments. We were fortunate in obtaining a sufficient quantity of radioactive isotopes of molybdenum (Mo^{93} and Mo^{99}) of high enough specific activity to permit short time plant absorption experiments with molybdenum levels of physiological significance. The high specific activity was attained through transmutation of zirconium by alpha bombardment. With the material available, it was possible to conduct absorption experiments using a single microgram of molybdenum for an entire culture supporting one to three vigorously growing tomato plants three to six weeks of age.

A preliminary report of the essential features of these experiments with radioactive molybdenum isotopes has been published elsewhere [28]). It was found that molybdenum uptake from single salt solutions was stimulated by phosphate ions to a greater extent than any other ion tested in the group consisting of Ca^{++}, Mg^{++}, NH_4^+, K^+, Na^+, $H_2PO_4^-$, $SO_4^=$, NO_3^-, and Cl^-. Moreover sulfate ions appeared to interfere with molybdate absorption at least by comparison with phosphate, nitrate or chloride ions. Typical data secured from a 24 hour molybdenum absorption experiment *) given in Table IV illustrates these points.

*) It should be noted that when conducting experiments with tagged elements it is necessary to know the total amount of the element present in the system capable of diluting the radioactive isotope by isotopic exchange. If, for example, one microgram of molybdenum were to be inadvertently contained in the culture solution in addition to the one added intentionally, the amount of molybdenum estimated from measurements of radioactivity would be only one half the true value. By use of the colorimetric stannous chloride thiocyanate extraction method (24) and extraction of pentavalent molybdenum thiocyanate in 3 ml of iso-amyl alcohol, one microgram of molybdenum can be estimated within $\pm$ 10%. Direct analysis of the molar salt solutions used to prepare the culture showed less than .5 microgram of molybdenum in 25 ml of the stock salt solutions of which 1 ml was used in preparing the culture. The pyrex distilled water prepared in our

Experimental method of short time absorption experiments with molybdate.

Twelve different salts, 0.005 Normal with respect to the cation, were placed in separate quart jars used as absorption vessels. Each culture solution of 800 ml contained one microgram of mlybdenum as molybdate tagged with the radioactive molybdenum isotopes Mo^{93} and Mo^{99}. Samples of the original radioactive material were set aside as standards for subsequent radioactivity assays of the various plant fractions. Marglobe tomatoes previously grown for three weeks in the same large culture tank in a complete nutrient medium were transferred to the one quarts jars for the absorption experiments. The average fresh weight of the plants was 8.8 grams but with individual variations ranging from 7.5 to 12.2 grams. The plants were chosen at random for the individual treatments. Oxygen was supplied by forcing air through sintered pyrex glass aerators. The absorption period began at 4 P.M. of one day and lasted to 4 P.M. of the next. Although the experiments of table IV*a* were conducted on the 27th and 28th of January, the plants had previously had the benefit of good growing weather, and on the day of the absorption experiment, warm sunny weather prevailed. Thus conditions conducive to transpiration were in effect.

After harvesting the plants, they were separated into blade, stem and root fractions, fresh weights were taken, and the several fractions were ashed and assayed for radioactive molybdenum. The concentration of molybdenum referred to the fresh weight of plant material gave more consistent comparative data than when referred to the total amounts of molybdenum absorbed per plant. Because the solutions were not well buffered growing plants caused rapid shifts in pH. Therefore, pH values were measured before and after the absorption period and are given in table V.

Data shown in Table IV were secured from 15 separate culture solutions, each having a single plant. The concentrations of radioactively tagged molybdenum found in the several treatments vary greatly, but even so, certain points of consistency were revealed. Of particular note was that in each instance where sulfate salts were used, the lowest molybdenum concentrations were found in stems and blades, and wherever phosphate salts were used, the highest amounts of molybdenum were found in stems and blades. Root absorbed molybdenum was higher than for tops of plants, and did not reflect similar differences. Thus, the highest degree of contrast is

laboratories for micronutrient cultures is known to supply less than .005 micrograms per liter, as has been determined by numerous plant culture experiments. Thus the molybdenum contamination in the culture solution used in these experiments was shown chemically to be less than .02 microgram, that is, less than 2% of the amount added for the absorption experiments.

shown by the molybdenum absorbed and translocated in the presence of phosphates and sulfates as may be seen in the molybdenum concentrations of stems and blades in lines 3 and 4 of Table IV.

TABLE IV

Molybdenum Absorbed in 24 Hours from Single Salt and Complete Cultures Initially Containing 1 Microgram of Radioactively Tagged Mo (Absorption calculated as parts per billion — Fresh weight)

Plant fraction	Blades			Stems			Roots			Percent of tagged MoO_4 removed from culture soln. in 24 h		
Cation/Anion	NH_4^+	K^+	Ca^{++}	NH_4^+	K^+	Ca^{++}	NH_4^+	K^+	Ca^{++}	NH_4^+	K^+	Ca^{++}
1. NO_3^-	27	17	33	2.9	4.1	10.4	44	16	31	23	11	18
2. Cl^-	21	7.4	11	4.2	1.4	7.8	7.0	34	10	13	9.3	11
3. $H_2PO_4^-$	39	97	44	13	21	21	60	86	86	37	61	34
4. $SO_4^=$	4.1	1.0	5.0	0.2	0.6	1.0	65	34	85	15	7.0	20
5. *0.1 strength culture solution			5.4			4.5			48			16

*0.1 strength Hoagland's culture solution, i.e. in m. eq. per liter Ca-10, Mg-0.4, K-0.6, NO_3-[illegible] SO_4-0.4, H_2PO_4-0.1.

On the assumption that phosphates stimulated molybdenum uptake it also appeared that sulfate ions depressed translocation from roots to the upper parts of plants.

Since the total accumulation of molybdenum by roots in the presence of either phosphate or sulfate salts was not significantly different, these data further suggested direct competition between sulfate and molybdate ions within channels through which divalent anions are transferred from roots to the conducting system of the plants.

Further evidence of analagous mechanisms of accumulation of sulfates and molybdates is to be found in the radio-autographs reported by Stout and Meagher[28]) and by Fried[14]). Stout and Meagher have called attention to the distinctly different pattern of distribution of absorbed molybdates from the patterns of other absorbed nutrient ions such as potassium, phosphorus, manganese, and zinc. They found that molybdenum was preferentially accumulated in interveinal areas of leaves whereas the other nutrient ions presented quite an opposite picture; namely, that they were preferentially absorbed in the midribs and veins.

Fried[14]) has found precisely the same pattern of distribution of absorbed sulfates in alfalfa as Stout and Meagher discovered for absorbed molybdate with the tomato plant. Moreover, Fried calls attention to the fact that the distribution pattern of absorbed

phosphates is directly opposite to that of sulfates in as much as his radioautographs showed a concentration of phosphorus in the transporting system of the alfalfa plant.

TABLE V

pH Values of Culture Solutions Before and After the 24 Hour Absorption Experiment of Table IV

Cation	NH_4^+		K^+		Ca^{++}	
	pH		pH		pH	
	before	after	before	after	before	after
NO_3^-	5.7	5.5	5.7	6.3	5.7	6.4
Cl^-	5.5	4.6	5.8	6.2	5.8	6.3
$H_2PO_4^-$	4.9	5.0	4.9	4.8	3.6	3.8
$SO_4^=$	5.5	4.4	5.7	5.6	5.6	5.6

0.1 strength culture solution, pH before absorption 5.5, pH after absorption 5.0.

The pH values of the culture solutions (Table V) showed the calcium phosphate solution to be more acid than the others. Also since some of the salt solutions were unbuffered, sizeable changes in reaction occurred in some of them. For example with NH_4Cl and $CaCl_2$, at the beginning of the absorption period, the pH of the NH_4Cl solution was 5.5 and the $CaCl_2$ solution was 5.8. After 24 hours they were found to be respectively pH 4.6 and 6.3. Because of such discrepancies in controllability of hydrogen ion concentrations in these experiments, others were undertaken for the purpose of securely establishing the interrelations between molybdates, phosphates and sulfate as they appeared to be suggested in the data of Table IV.

In order to obtain more substantial evidence of the indicated phosphate effect, it was decided to investigate separately first, the influence of the hydrogen ion concentration of the culture solution on the absorption of molybdenum from solutions containing phosphate and molybdate ions; second, the quantitative interrelations between phosphate concentration and molybdate absorption over wider ranges of phosphate concentrations at a fixed molybdenum supply; and third, the influence of sulfate ion concentration on molybdenum absorption at a fixed molybdenum supply. Also implications of interrelations between molybdate and sulfate, or molybdate and phosphate revealed in short time absorption studies were to be tested over much longer growth periods, preferably with both culture solutions and soils. All subsequent experiments have supplied

corroborative evidence of enhanced molybdenum uptake with increasing phosphate levels, and further evidence was obtained showing the opposite effect with increasing levels of sulfate ions.

Effect of hydrogen ion concentrations on molybdenum absorption by plants from culture solutions containing phosphate. Increased molybdenum adsorption by plants has been associated with increasing degree of soil alkalinity by Anderson and Oertel[3]), Stevens and Oertel[25]) and Piper[21]). They have shown that making soils less acid results in increased molybdenum absorption by plants, and have interpreted their findings as meaning that exchangeable molybdenum is released from soil materials as they are rendered less acid. Such an interpretation is proper in view of known properties of molybdate as an exchangeable anion.

Since all soils or culture solutions capable of supporting plants contain phosphates, it was decided that measurements of the hydrogen ion effect in the presence of phosphates should be informative of general situations with growing plants. In our work with culture solutions it was expected that molybdenum absorption would be found to vary with the hydrogen ion concentration since the solution pH determines the dominant type of phosphate ions in solution.

In the following experiment involving an absorption period of 6 hours the radioactive isotope used was mostly Mo^{93} of half life 6.7 hours. Procedures were the same as described earlier in this paper, with some minor exceptions to be noted. Marglobe tomatoes were first grown for two weeks in a single large tank. Three plants were later transferred to each of a series of absorption vessels containing potassium hydrogen phosphate buffers .0022 molar with respect to PO_4, made up to cover the range of pH 3.5 to pH 8.5. Each culture was provided with 1 microgram of the radioactivity tagged molybdenum, and three replications were made of each culture.

After the absorption period the plants were harvested, separated into tops and roots, assayed for radioactive molybdenum. The pH of the culture solutions was not controlled during the absorption period, but the change in reaction for the entire period was determined from pH measurements of the cultures immediately after the plants were removed. Solutions of pH 6 remained relatively constant. Cultures either more acid or more alkaline shifted toward pH 6 as a result of the action of absorbing plants.

Data showing the absorption of molybdenum by the tops of the plants as a function of culture solution pH are given in Figure 1. Accumulations by the roots are shown in Figure 2. The amount of molybdenum absorbed is given as parts per billion fresh weight for each replicated culture. Changes of the pH of the culture solution during the absorption period are indicated by the length of the arrows drawn parallel with the abscissa. The circles on the arrow indicate the pH of the culture solution as it was at the beginning of the absorption period, and the head of the arrow the final pH. The length and direction of the arrow, therefore, shows the magnitude and direction of the shift in pH during the period of absorption.

The effect of acid reactions on molybdenum absorption is very large, the plants of the two most acid solutions having absorbed in the order of 5 times the amount of plants in the two most alkaline solutions. In this experiment, concentration of radioactive molybdenum found in plants was higher than that of the culture solution. Thus molybdenum accumulation took place against a concentration gradient, the molybdenum concentration of the culture solution initially being one part per billion, and the concentration in the plants from 1 to 8 parts per billion depending upon the particular culture solution. Molybdenum concentrations found in the roots were very much higher than in the tops — from 10 to several hundred parts per billion.

It is manifest that, under the conditions of this experiment, acid reactions favor molybdenum absorption by roots, and also the amount transported into the tops of the plants. The most rapid rise in the efficiency of absorption by tops lies within the region of pH 4.5 to pH 5.5. No reason for this behavior can be given from these data, but it is observed that the region of most rapid increase in absorption efficiency also corresponds to the pH values of solutions where the H_2PO_4 ion predominates. One might therefore speculate that the influence of phosphates in enhancing molybdenum uptake would be more intense where the phosphates were in the form of $H_2PO_4^-$.

Although these data show that plants are capable of extracting molybdenum from acid solutions much more efficiently than from alkaline ones, this fact is not contradictory to the findings of other workers in experiments with soils who have reported [3] [21] [25] higher molybdenum accumulations by plants as a result of adding lime to

acid soils. It can be postulated that the release of molybdenum fixed by soil colloids must more than compensate for the inhibitory influence of alkaline solutions as shown graphically in figures 1 and 2.

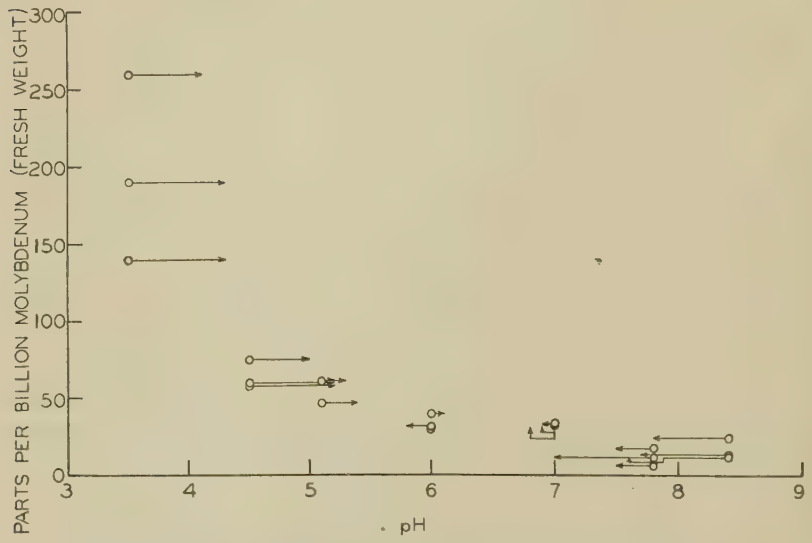

Fig. 1. Effect of pH on molybdenum absorbed and translocated to shoots of plants from solutions of .0022 *M* PO_4 and 1.1 p.p.b. Mo as MoO_4.

Effect of Phosphate Concentration of Culture Solutions on Molybdenum Absorption by Plants. Since the earlier experiments with soils as well as culture solutions suggested that increased phosphate levels could be specifically associated with higher molybdenum absorption, we wished to obtain more information as to the different phosphate concentrations over which the observed interrelation with molybdenum absorption would hold. In the following experiment, culture solutions of different concentrations of KH_2PO_4 were used. The experimental procedure was analogous to the short time absorption experiments using radioactive molybdenum already described. Seedlings of marglobe tomatoes were transplanted to a single tank and grown for three weeks without supplemental additions of micronutrients. For the absorption experiment the plants were transferred to quart mason jars provided with aerators. Each jar contained 1 microgram of radioactivity tagged molybdenum, but the phosphate levels were made to 0, 5.0, 25, and 125 parts per million PO_4 by adding

KH_2PO_4. There were three replicate cultures for each level of KH_2PO_4.

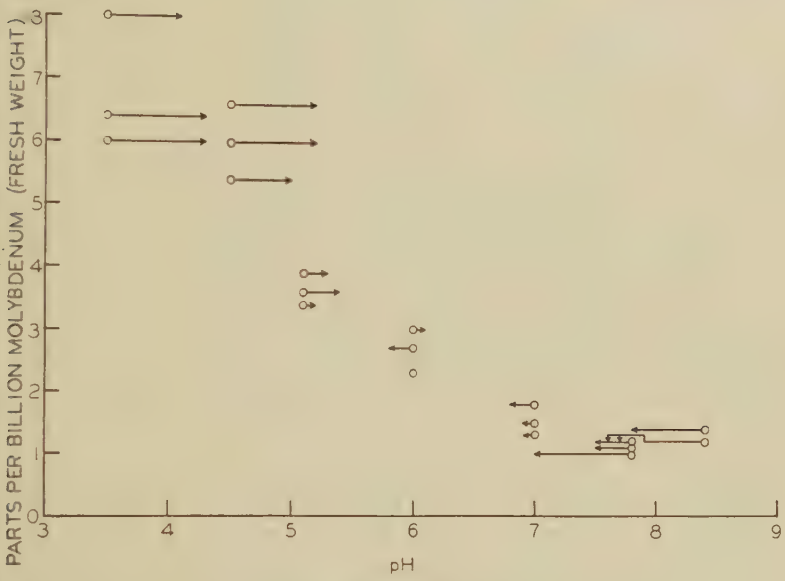

Fig. 2. Effect of pH on molybdenum absorbed and retained by roots from solutions of .0022 *M* PO_4.

After an absorption period of 24 hours, the plants were harvested and assayed for radioactivity from which absorbed molybdenum was calculated. Mean values of molybdenum found in blades, stems and roots are given in Table VI. Increased molybdenum concentrations were found in all plant fractions as a result of adding KH_2PO_4. Moreover, the increase was reflected at each higher level of potassium phosphate.

The accumulations of molybdenum in the blades of each plant in the experiment are shown graphically in Figure 3. Triangular points on the graph represent single culture and circles, the mean concentrations found for identical treatments. The line in Figure 3 was arbitrarily inserted as representing a linear relationship between the concentration of phosphate in the culture solution and molybdenum uptake, and was drawn between the mean molybdenum concentrations at the two terminal treatments. We attach no significance to the linearity of the relationship but do wish to emphasize the wide

TABLE VI

Effect of Phosphate Concentration of Culture Solutions on Molybdenum [1]) Uptake by Plants [2])

Treatment p.p.m. PO_4 in culture solution	Parts per billion fresh weight [3])		
	Blades	Stems	Roots
None	2.5	1.4	10
5	3.2	1.85 [4])	13.5
25	11.3	6.0	20
125	35	14	41

[1]) 1 microgram molybdenum tagged with Mo^{93}, Mo^{99} added to each culture solution of 800 cc.

[2]) Marglobe tomatoes grown three weeks prior to the absorption experiment in a culture solution with no micronutrient supplement.

[3]) Calculated from assays for radioactivity, each figure given is the mean of three separate plants.

[4]) One sample lost: value indicated is the average of 2 samples.

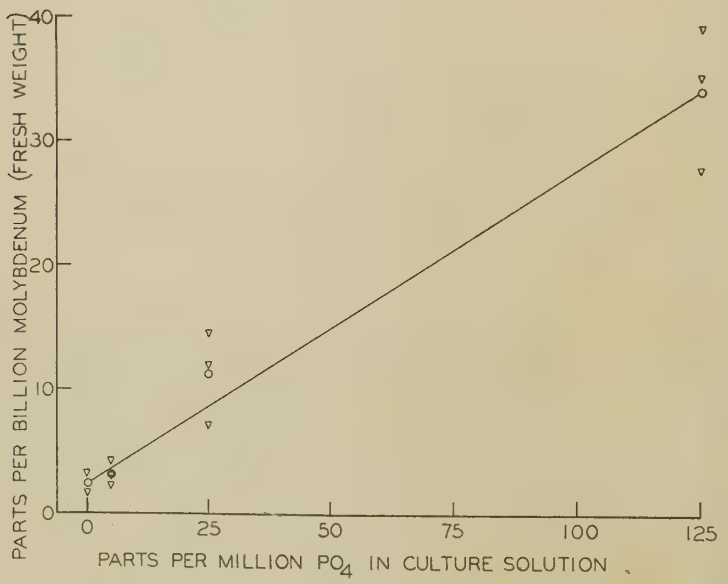

Fig. 3. The influence of phosphate concentration on molybdenum absorption as shown by tomato plants in a 24 hour absorption period. Solutions initially contained 1 part per billion radioactively tagged molybdenum as molybdate ion.

range in phosphate concentrations over which the molybdenum-phosphate interrelation is operative.

As with other short time absorption experiments, it was found that

absorption of molybdenum by roots was not significantly influenced by the composition of the culture solution, but the amount of molybdenum translocated to the upper parts of the plant was greatly different.

It was earlier pointed out that experimental artifacts might result from isotopic dilution of the radioactive isotopes with inadvertantly included non-active molybdenum isotopes. This, however, may be excluded from consideration in these experiments since the only regular variable introduced in the experiment was the amount of KH_2PO_4 added. Any effect of isotopic dilution with molybdenum impurities in the phosphate would lead to results diametrically opposite to those obtained.

The response observed is attributed to phosphates and not to potassium added concomitantly, since other experiments (Table IV) have shown no measurable effect of different cations including potassium, ammonium and calcium.

Phosphate levels of culture solutions with respect to molybdenum absorption by plants grown on culture solutions to maturity. If enhanced molybdate absorption induced by phosphates as shown in the above section for an absorption period of 24 hours were to have general significance in plant nutrition it should also be evidenced by tomato plants grown to maturity. We were able to check this point by analyzing plant parts made available to us from another study of Arnon and Hoagland [5]. In their studies in connection with the mineral composition of tomato plants they had occasion to grow plants to the fruiting stage at two different levels of phosphate supply.

It appeared that one of their experiments designed to determine the influence of phosphate on the mineral nutrition of bearing tomato plants would also serve admirably as a means of determining the influence of phosphate on molybdenum absorption. Their plants had been grown for 5 weeks on a complete culture solution at which time the first flowers had emerged. Plants were then selected for uniformity and divided into groups of 24 in other culture solutions. Some were allowed to set fruit and others were deflowered. Both kinds of plants were further grown on culture solutions with an without phosphate. The only other variation in the compositions of the culture solutions was with respect to sulfate which was used to replace

phosphate in the minus phosphate cultures. However, the total concentration of sulfate in the two culture solutions was not greatly different, being 14 m. eq. per liter in the high phosphate culture, and 16 m. eq. per liter in the minus phosphate culture. It was reasoned that the specific influence of sulfate on the absorption of molybdate should thus be minimized and even if molybdenum absorption directly decreased in proportion to sulfate concentration, the expected decrease should not be greater than $^1/_7$ or approximately 15%. Phosphate stored by the plants during their first five weeks of growth was sufficient to allow subsequent fruiting on the minus phosphate cultures, although their total growth was approximately one fifth that of the plants having a high phosphorus supply. The deflowered plants produced approximately twice the vegetative growth of those allowed to set fruit. Thus, comparisons of molybdenum absorption within this particular set of plants should provide information as to the general extent of the phosphate molybdate interrelation over a wide range of variability with respect to plant growth under different nutritional, and physiological states.

All similar plant materials available which could be paired between high and low phosphate culture solutions on a given harvest date were analyzed for molybdenum. The molybdenum contents of the several plant parts are given in table VII.

Without exception, it is shown in Table VII that comparable plant parts grown on solutions containing 200 p.p.m. phosphate have higher concentrations of molybdenum than those grown part of the time on cultures without phosphate. Averaging the concentrations of molybdenum found in leaves, without regard to position along the stem, condition of fruiting of the plant, or harvest date, the mean concentration was 1.85 p.p.m. molybdenum for leaves from plants grown on high phosphate cultures and 0.85 p.p.m. molybdenum for the low phosphate cultures. Corresponding averages for molybdenum concentrations of stem material were 1.20 and 0.54 p.p.m. molybdenum respectively for the high and low phosphate cultures. The increased molybdenum concentrations shown to be associated with higher phosphate concentrations are highly significant, exceeding the .1% level for both leaves and stems.

Another point of interest in the analyses shown in Table VII was the lower molybdenum content of stems as compared to leaves, which provided corroborative evidence earlier established with radio

TABLE VII

Relation of the Phosphate Level to Molybdenum Uptake by Tomato Plants Grown to Maturity in Culture Solution

Harvest Date	Differential treatment other than nutritional	Plant Part	Position [1] on Plant	Molybdenum p.p.m. dry weight	
				High PO_4 [2]	Low PO_4 [3]
5/22/42	Fruitful	Leaves	2	1.9	0.8
			4	2.0	1.1
		Stems	4	1.2	0.6
	Deflowered	Leaves	6	1.8	1.1
		Stems	6	1.3	0.6
			5	1.3	0.4
7/10/42	Fruitful	Leaves	1	2.0	0.5
			3	2.1	0.7
		Stems	3	1.1	0.5
			22	1.1	0.6
	Deflowered	Leaves	2 and 3	1.3	0.9
Mean concentration in leaves (all data)				1.85	0.85*** [4]
Mean concentration in stems (all data)				1.20	0.54*** [5]

[1] Descending from top of plant.

[2] Phosphate in culture solution 200 p.p.m.

[3] Phosphate in culture solution none after first 5 weeks.

[4] Comparing low with high PO_4 treatments for significance of increased molybdenum concentration in leaves. $t = 6.59$, 10 degrees of freedom. Significance exceeds the .1% level.

[5] Comparing low with high PO_4 treatments for significance of increased molybdenum concentrations in stems. $t = 11.0$, 8 degrees of freedom. Significance exceeds the .1% level.

autographs of plants having absorbed Mo^{93} and Mo^{99} [28]) that molybdenum, unlike other essential elements absorbed by roots is less actively accumulated by cells adjacent to the transpiration stream, and is thus deposited in relatively higher concentrations in the leaves.

Effect of sulfate on molybdenum absorption in the presence of phosphate ion. Whereas earlier experiments were conducted with single salt solutions the following one was designed to permit observation of the influence of sulfate ion on the absorption of molybdate from solutions also containing phosphates. As in our earlier short time absorption experiments, high specific activity radioisotopes were used in order that quantitative analysis could be made of sub-micro-

gram amounts. The total amount of tagged molybdenum added to the culture solutions of 800 ml was one microgram.

Marglobe tomato plants were grown in a single culture of one tenth the usual strength for three weeks prior to the experiment. Although the plants had the benefit of excellent growing weather and appeared healthy, they varied considerably in size as indicated by their fresh weights (Table VIII). No attempt was made to choose plants of uni-

TABLE VIII

Effect of Sulphate on Mo[1]) Absorption from Solutions of KH_2PO_4. Absorption Period 41 Hours

Culture Solution Numbers	Section	Treatment	Mo, Parts per billion (fresh weight of plant)						Mean	σ_x[5])	t[6])
			a	*b*	*c*	*d*	*e*	*f*			
1–6[2])	Blades	1. PO_4	9.9	14.4	9.3	10.7	6.6	15.1	11.0	1.31	4.10**
7–12[3])	,,	2. $PO_4 + SO_4$[3])	4.7	5.1	5.5	5.9	5.3	6.6	5.5	0.27	
1–6	Stems	1. PO_4	10.6	13.3	21.3	6.7	3.4	8.7	10.7	2.53	3.35**
7–12	,,	2. $PO_4 + SO_4$	2.1	2.6	2.1	2.2	1.7	2.6	2.2	0.14	
1–6	Roots	1. PO_4	22	22	20	22	16	18	20.1	1.03	1.66 n.s.
7–12	,,	2. $PO_4 + SO_4$	18	13	23	18	12	17	16.8	1.62	
		Fresh weight of plants — grams[4])									
1–6			6.2	5.6	6.7	11.0	8.9	12.1	8.4	1.09	
7–12			5.1	8.0	6.7	7.3	18.8	6.4	8.7	2.06	0.13 n.s.
		pH of culture solutions after absorption period									
1–6		PO_4	5.5	5.4	5.4	5.4	5.5	5.2			
7–12		$PO_4 + SO_4$	5.3	5.4	5.3	5.3	5.2	5.3			

[1]) Molybdenum tagged with Mo^{99}. 1 microgram Mo added to each culture as Na_2MoO_4.

[2]) 125 p.p.m. PO_4, added as KH_2PO_4 to all cultures, Columns *a*, *b*, *c*, *d*, *e*, *f*, correspond to solution culture numbers 1, 2, 3, 4, 5 and 6 respectively.

[3]) 125 p.p.m. PO_4 plus 200 p.p.m. SO_4 added as KH_2PO_4 and K_2SO_4 to all cultures, Columns *a*, *b*, *c*, *d*, *e*, *f*, correspond to solution culture numbers 7, 8, 9, 10, 11 and 12 respectively.

[4]) Fresh weights were taken after harvest, total weight included roots.

[5]) σ_x = standard error of the mean.

[6]) Comparison between treatments 1 and 2 for significance of differences in molybdenum concentrations. Student's t values for 10 degrees of freedom, at 10% level, $t = 1.81$; at 5% level = 2.23 at 1% level = 3.17.

The total amount of molybdenum adsorbed per plant was highly variable. From these data it is apparent that the amount of molybdenum accumulated by a plant is nearly a direct function of its size as determined by weight.

form size but 12 plants were selected from the stock culture tanks at random and transferred to 12 quart jars provided with means of aeration. Two treatments were used, one being phosphate alone, and the other phosphate plus sulfate. There were six replicate cultures

for each treatment. Solutions received 1 microgram of tagged molybdenum and sufficient KH_2PO_4 to make them 125 parts per million with respect to PO_4. The phosphate plus sulfate cultures were made to contain 200 parts per million SO_4 added as K_2SO_4.

Since the relatively large amount of KH_2PO_4 in the culture solutions acted as a buffer, the absorption period was extended to 41 hours (5 : 30 p.m. 4-2-48 to 10 : 30 A.M. 5-2-48). After the absorption period the plants were harvested, weighed, and separated into blade, stem and root fractions. Each fraction was assayed for absorbed molybdenum by means of the radioactivity of Mo^{99}. Results of the analyses are given in Table VIII.

The lowest molybdenum concentration found in the blades of a plant absorbing from a solution of KH_2PO_4 free from sulfate was 6.6 p.p.b. (col. *e*). The highest molybdenum concentration found in the blades of a plant absorbing from a solution containing added K_2SO_4 was also 6.6 p.p.b. (col. *f*). All other possible pairings between the two types of culture solutions showed molybdenum absorption to be depressed when sulfate was included.

Analogous comparisons between the two treatments referred to the plant stems show, without exception, that addition of K_2SO_4 reduced the concentration of molybdenum found in the stems.

Statistical treatment of the molybdenum concentrations in either the blades or stems, shows that the observed repression of molybdate absorption is highly significant, being above the 1% level for the blades, and above the 5% level for the stems.

Although this experiment in itself does not distinguish between potassium and sulfate as the factor responsible for suppression of molybdate absorption, the data of Table IV and of other experiments already discussed, make it evident that if potassium does not effect molybdate absorption, its influence is small relative to that exercised by sulfate.

Depression of Molybdenum Absorption from Soils Treated with Calcium Sulfate. Having studied the role of phosphates and sulfates in relation to molybdenum absorption from culture solutions it was thought that some gross corroborative test of the principles revealed should be made using the much more complex soil medium. If the observed influences of phosphates and sulfates were generally characteristic of the physiology of plants, it could be expected that

higher soil phosphate levels should give rise to greater molybdenum accumulation by plants. Conversely, any soil factors contributing to lower availability of phosphate should, for a given level of molybdenum, result in a lower molybdenum uptake. It should also be expected that higher soil sulfate levels should result in decreased molybdenum absorption by plants.

It was reasoned that both factors having been found to influence molybdenum absorption by plants would be simultaneously invoked if calcium sulfate were to be added to a neutral soil.

Reasons for expecting additions of calcium sulfate to suppress absorption of molybdenum through indirectly decreasing soluble soil phosphate of a neutral or slightly alkaline soil were as follows: In neutral or slightly alkaline soils, soluble phosphates in the soil solution are largely determined by the amount of soluble calcium ions present. Therefore, addition of a soluble calcium salt to such a soil should result in directly lowering the concentration of soil solution phosphate. B u r d [10]) has particularly emphasized this point in experiments showing a reciprocal relationship between the calcium and the phosphate concentrations of the diluted solutions coming from soil columns after the soil solution proper has been displaced by water. A second reason for expecting decreased molybdenum uptake upon supplementing soils with calcium sulfate was simply that the soil sulfate level would be thereby increased. These expectations have been entirely borne out as will be shown in the data of Table X. In order to minimize complications due to variations in growth that might result from the several treatments a fertile soil was selected for the experiment, namely, Yolo cilty clay loam. The soil was regarded as highly fertile for agricultural crops. Also pot tests had shown no increased production in the first crop when fertilized with N, P or K*.

Chemical studies of the effect of additions of $CaSO_4.2H_2O$ *and* KNO_3 *to the water soluble calcium, phosphate and sulfate of Yolo silty clay loam.* Other physical and chemical data characterizing the soil were: moisture equivalent 24.6; cation exchange capacity 21.8 m. eq. per 100 g; and NH_4Ac extractable cations being, Ca 14.0, Mg 8.2, K .78, and Na .45 m. eq. per 100 g. Water soluble nitrate was 173 p.p.m.

*) This soil was supplied to us by Dr. A. U l r i c h who had used it in connection with pot tests with sugar beet production.

The comparative figure for phosphate supplying power of this soil as given by Bingham's water extraction method [8]) was .20 p.p.m. PO_4 in the 1 : 10 water extract. According to Bingham's criteria, 82 percent of randomly selected soils showing this amount of water soluble phosphate could be expected to be phosphate deficient. It must be assumed that although the phosphate supplying power of this soil was low, it was not limiting. Further testimony of the high level of fertility of this soil is given in the uniformly high yields obtained with the first crop of tomatoes as shown in column *e* of Table X.

TABLE IX

Changes in the Calcium, Phosphate, and Sulfate Concentrations of 1 : 1 Water Extracts at Various Levels of $CaSO_4$ and KNO_3 Added to a Yolo Silty Clay Loam

Treatment	Crop numbers to which treatment apply	Salt added		Equivalents per million parts of solution in 1 : 1 water extracts			Product Ca^{++} X $HPO_4^{=}$ Moles/l $\times 10^9$	Water sol. PO_4 in a 1 : 10 extract p.p.m.	pH of 1 : 1 suspension	g dry wt. tomatoes 1st crop
		p.p.m.	Lb per acre	Ca^{++}	$HPO_4^{=} \times 10^3$	$SO_4^{=}$				
a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*
1	1,2	None	None	4.40	2.47	0.43	1.55	0.20	6.8	138
3	1	100 p.p.m.								
		$CaSO_4.2H_2O$	196	5.04	2.16	0.96	1.55	0.20	6.8	134
4,5	1,2	400 p.p.m.								
		$CaSO_4.2H_2O$	284	6.90	1.96	2.82	2.26	0.17	6.9	133
6	2	2000 p.p.m.								
		$CaSO_4.2H_2O$	3920	18.24	1.71	12.31	3.74	0.11	6.7	
7	1	*50 p.p.m. N	64.5 N	5.50	2.08	0.36	1.63	0.19	6.8	136
8	2	*152 p.p.m. N	196 N	8.54	1.84	0.32	2.24	0.15	6.8	
9	1	*200 p.p.m. N	258 N	10.10	1.65	0.27	2.38	0.15	6.8	136
10	2	*455 p.p.m. N	586 N	16.80	1.48	0.35	2.08	0.13	6.5	

*) Added as KNO_3.

In order to determine changes in the water soluble sulfates and phosphates induced by the additions of $CaSO_4.2H_2O$ and KNO_3, separate samples of soil were used. After treatment, 1 : 1 water extracts were analyzed. Results of the analyses are given in Table IX.

As would be expected, additions of $CaSO_4.2H_2O$, raised the amount of water soluble calcium and sulfate as shown in Table IX, columns *e* and *g*. With increasing water soluble calcium, phosphate concentrations in the water extract was reduced from 0.20 to 0.11

p.p.m. (column *i*). It was also noted that the solubility product $[Ca^{++}]\ [HPO_4^{=}]$, increased from 1.55 to 3.74×10^9, when 2000 p.p.m. $CaSO_4.2H_2O$ were added to the soil. It is suspected that this was due to release of some exchangeable hydrogen even though the suspension pH was not materially effected within the degree of accuracy of the pH measurements. Increasing additions of KNO_3 also resulted in corresponding reduced water soluble phosphate. Reasons for the later are to be found in analyses of water soluble calcium shown in column *e*. It is seen that the added potassium replaced calcium from the exchange complex. Also upon addition of KNO_3 some exchangeable hydrogen may have been released, as evidenced by an increase in the product $[Ca^{++}]\ [HPO_4^{=}]$. (Column *h*, Table IX).

Procedure for determination of the effect of adding $CaSO_4.2H_2O$ and KNO_3 to soils on the molybdenum absorption by plants. Seven kilograms of Yolo silty clay loam were placed in 2 gallon stoneware crocks, each having an area of 7.75×10^{-6} acres and a soil depth of 8 inches. Four separate crocks were provided for each treatment. Additions of gypsum and potassium nitrate were made by dry mixing the finely ground salts. Seeds were planted in the crocks, and after they had germinated molybdenum was added to the surface as a solution of Na_2MoO_4.

The ten different soil treatments were distributed over two different crops as shown in columns *a*, *b*, *c* and *d* of table X. The first crop received six treatments, two of them (treatments 3 and 5) involving additions of $CaSO_4.2H_2O$, and two others (treatments 7 and 9) the addition of KNO_3. Treatment 1 was the control and treatment 2 had only Na_2MoO_4 added. Between the first and second crops, some crocks having received $CaSO_4.2H_2O$ or KNO_3, were supplemented with further additions of the respective salts. Thus the crocks of treatments 3 and 5 receiving $CaSO_4.2H_2O$ before the first crop became treatments 4 and 6 for the second crop at higher levels of added $CaSO_4.2H_2O$. Similarly, treatments 7 and 9 of the first crop, upon further addition of KNO_3 became treatments 8 and 10 for the second crop.

The plants were grown in the open, each crock being thinned to five tomato plants or four pea plants. Two successive crops were grown. Crop 1 was planted April 7 and harvested July 10, 1948, and

crop 2 was planted October 6, 1948, and harvested January 3, 1949. A second crop of tomatoes was much inhibited in growth during the colder weather and was finally lost by freezing. After harvest, the plants from each crock were dried, weighed and analyzed for molybdenum content as single samples. Molybdenum contents of the plant materials are shown in Table X.

TABLE X

ression of Molybdenum Absorption of Tomato and Pea Plants by Additions of $CaSO_4$ to a Fertile Soil

Additions to Soil [1]				First Crop [2] Tomatoes			First Crop Peas [2]			Second Crop [3] Peas	
				Yield			Yield				
Material added in large amount	Amt. added as p.p.m. dry soil	Amt. added as lbs per acre	Mo [1] treatment	Total g dry wt.	tons per acre	p.p.m. Mo	Total g dry wt.	tons per acre	p.p.m. Mo	Yield total g dry wt.	p.p.m. Mo
a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*
None	None	None	None	138	4.9	1.25	111.2	4.0	1.3	29.8	1.7
None	None	None	+	115	4.1	5.25	87.3	3.1	12.8	32.5[3]	16.0
$CaSO_4.2H_2O$	100	196	+	134		3.52	110.0	3.9	8.05		
$CaSO_4.2H_2O$	400	784	+		4.8					46.8	6.0
$CaSO_4.2H_2O$	400	784	+	133	4.7	2.45	93.5	3.3	5.7		
$CaSO_4.2H_2O$	2000	3920	+							33.6	2.75
*N	50	64.5	+	136	4.8	4.7	99.5	3.7	9.4		
*N	152	146	+							44.4	8.8
*N	200	258	+	136	4.8	4.8	98.6	3.7	7.1		
*N	455	586	+							31.8	5.5
yield ± Standard Error of the Mean				132.0 ± 3.0 g			100.2 ± 3.9 g			36.5 ± 9.2 g	

l treatments except treatment 1 received 1.5 pounds molybdenum per acre, as Na_2MoO_4.
anted April 7, 1948, Harvested July 10, 1948.
anted Oct. 6, 1948, Harvested Jan. 3, 1949: Second planting of peas in same soil used for first crop of peas.
cond crop was grown on same set of pots having 196 lb/acre $CaSO_4.2H_2O$ during first cropping.
econd crop was grown on same set of pots having 784 lb/acre $CaSO_4.2H_2O$ during first cropping.
otal N added for 2 crops. Second crop was grown on same set of pots, receiving 64 N acre for first crop.
otal N added for 2 crops: Second crop was grown on same set of pots receiving 25 N for the first crop.
ne pot was lost from the set. Weights of remaining 3 pots multiplied by 1.33 to obtain comparable
r set.
dded as KNO_3.

Discussion of molybdenum absorption by plants as influenced by addition of $CaSO_4.2H_2O$ and KNO_3. In treatments 1 and 2, table X, it is shown that the addition of 1.5 pounds molybdenum per acre raised the molybdenum content of the tomatoes from 1.25 p.p.m. to 5.25 p.p.m. and that of peas from 1.3 to 12.8 p.p.m. The second crop

of peas were consistent with the first, the respective molybdenum concentrations being raised from 1.7 to 16.0 p.p.m.

However, addition of $CaSO_4.2H_2O$ (treatment 3) at the rate of 196 pounds per acre, decreased the molybdenum content of tomatoes and peas in all crops. In the first crop the molybdenum in tomatoes (column *g*) was reduced from 5.25 to 3.52 p.p.m. and with peas from 12.8 to 8.05 p.p.m. (column *j*).

A further decrease in molybdenum absorption resulted from the higher rate of application of 784 pounds $CaSO_4$ per acre (treatment 5), tomatoes being reduced to 2.45 p.p.m., and peas to 5.7 p.p.m. The second crop consisting only of peas had the molybdenum content reduced from 16.0 to 6.0 p.p.m. (Column *l*) as a result of adding 400 lbs. $CaSO_4.2H_2O$ per acre (treatment 4). Also, consistent with the trend of all other comparative data, addition of 3920 lbs. $CaSO_4.2H_2O$ per acre (treatment 6) further decreased the molybdenum content of peas to 2.75 p.p.m. Within each crop, all differences in molybdenum content of plant materials resulting from the additions of $CaSO_4$ have been treated statistically and shown to be significant at the 5% level or better.

Significant reductions in molybdenum absorption were also affected by additions of KNO_3 but they were not as great as for equivalent amounts of $CaSO_4$ added to the soil. The reduction of molybdenum absorption by plants as a result of adding KNO_3 could be regarded as the result of competition between nitrate and molybdate. However, it was also shown in Table IX that additions of KNO_3 to the soil reduced water soluble phosphate, ostensibly through the suppression of the solubility of $CaHPO_4$ by calcium ions released by exchange with the potassium added as KNO_3. Therefore, it might also be deduced that the decreased level of soluble soil phosphate is compatible with a corresponding inhibition of molybdenum absorption as was indicated by studies with solution cultures discussed earlier.

Thus it has been shown that calcium sulfate added to soils exercised a marked depression of molybdenum accumulated by the two plant species, tomatoes and peas. Moreover, the degree of inhibition could be quantitatively assessed at different levels of added $CaSO_4.2H_2O$, ranging from the moderately low application of 196 pounds per acre to 3920 pounds per acre.

If, of course, cannot be specifically claimed that inhibition of

molybdenum absorption, shown in Table X can be entirely ascribed to competition between sulfates and molybdates and to the lower phosphate concentrations effected in the soil solution by adding calcium sulfate. It is of interest, however, that the predicted trend should be so nicely shown by an experiment designed to test the implications of the findings of laboratory studies of soil phosphates, coupled with short time plant absorption experiments involving submicrogram amounts of molybdenum. Since it has been established that increased levels of calcium sulfate in the soil suppress molybdenum absorption by plants any explanations of the phenomenon must account for the contribution of the sulfate and phosphate factors as well as others which might be involved.

Summary

It has been found that 1 pound per acre of molybdenum added to soils as Na_2MoO_4, is reflected in large increases in the molybdenum absorbed by plants. If, however, phosphate levels of the soil are increased at the same time, the molybdenum absorption is considerably enhanced, sometimes by as much as tenfold. Under conditions of intensive cropping and at high phosphate levels, added molybdenum is very rapidly removed, recoveries of 10 to 50% having been attained in a single crop.

Plants are capable of extracting molybdates from very low concentrations in culture solutions. From short time absorption experiments with radioactive molybdenum it has been shown that molybdates are absorbed from concentration of 1 part per billion of culture solution and are translocated to the upper parts of plants. Absorption is enhanced in the presence of phosphates and depressed in the presence of sulfates. Since molybdate ions are divalent and of the same size as sulfates, it may be assumed that these two ions compete directly for adsorption spots on the root during the first step of absorption. Enhanced uptake in the presence of phosphates however, is difficult to explain. At present we can only say that it is characteristic of plants since it has been observed under widely different circumstances, in short time absorption periods and in long term growth periods in culture solutions as well as in soils. Moreover, the effect of phosphates is diametrically opposite to that which would be expected from consideration of ordinary physical chemical interchanges between anions. Explanations for the effect must therefore be sought in biochemical processes involving the two ionic species.

Considering only the molybdate absorption from soil systems, it could be expected that added phosphates might exchange for soil adsorbed molybdenum and thus partially account for increased absorption of molybdenum by plants, analogous to the postulated release of soil molybdenum upon making soils alkaline by exchange of soil adsorbed molybdate for

hydroxyl ions. Though soil molybdate is fixed more strongly at acid reactions, it has been shown in culture solution studies that adsorption of molybdates by plants is greater from acid solutions than from solutions of neutral reactions. It has been reported that plants absorb greater amounts of molybdenum from acid soils upon liming. It must therefore be considered that when lime is added to such soils, the increased concentration of available soil molybdenum is more than sufficient to overcome the decreased ability of plants to absorb molybdate from increasingly alkaline media.

Interrelations between soil molybdenum and sulfate or phopshates are of such nature that important changes in the molybdenum content of plants may be brought about by additions of moderate quantities of phosphate fertilizers or gypsum likely to be used in ordinary fertilization practice. For example, it was shown that subterranean clover grown on an acid, phosphate deficient soil having been given one pound of molybdenum per acre contained an average of 6 p.p.m. molybdenum in the plants. Fertilization with 405 pounds P_2O_5 per acre increased the yield $2^1/_2$ times but even with the increased plant growth, the concentration of molybdenum in the plants was raised from 6 to 70 p.p.m.

Decreased molybdenum absorption by soil grown plants can be affected by lowering soluble phosphate and increasing soil sulfate levels. This was shown with a fertile soil of neutral reaction to which the addition of 196 pounds $CaSO_4.2H_2O$ per acre decreased the molybdenum content of pea plants from 12.8 to 8.05 p.p.m. and tomato plants from 5.25 to 3.52 p.p.m. molybdenum. Higher applications of 3920 lbs. $CaCO_4.2H_2O$ per acre further reduced the molybdenum content of peas from 16.0 to 2.75 p. p.m.

The experiments and findings so far discussed have brought out some of the important chemical features of soils and culture solutions governing molybdenum absorption by plants from levels of molybdenum supply adequate for their nutrition requirements. Another paper now in preparation deals with California soils which have produced molybdenum deficiency in a number of different species of crop plants.

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THE EFFECTS OF GLUCOSE ON IMPURE CULTURES OF NITRIFYING BACTERIA

by JANE MEIKLEJOHN

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

Introduction. In the course of obtaining pure cultures of *Nitrosomonas europaea* [6]), I had a number of crude cultures containing both ammonia-oxidising and nitrite-oxidising bacteria, together with at least seven non-nitrifying species. As glucose had been reported to be toxic to pure cultures of nitrifying bacteria in concentrations as low as 0.2–0.3 per cent, (II), I thought it would be interesting to see the effect of glucose, at about the same concentration, on mixed cultures.

Material and Methods. The cultures were serial transfers from a soil enrichment culture, growing at 25° in a liquid medium [6]), Medium B) containing 0.005 M $(NH_4)_2SO_4$. (140 mg Nitrogen per litre). The medium was sterilised by autoclaving in 20 ml portions in 250 ml conical flasks.

Glucose added to the medium was sterilised in two ways, filtration or autoclaving. A 3.6% (*w/v*) solution of A. R. Dextrose in distilled water was filtered through a Ford SB pad, and 2 ml added to 20 ml sterile medium, giving a 0.33% solution (approx. 0.02 M). Or enough A.R. Dextrose to make a 0.02 M glucose solution was added to the medium and autoclaved with it.

Replicate cultures (control and glucose-treated) were inoculated at the same time with 0.5 ml from a control culture in which nitrification had taken place. Plating of control cultures showed that they contained seven non-nitrifiers (a *Proactinomyces*, a *Micromonospora*, and five species of bacteria, two of which fermented glucose), as well as *Nitrosomonas* and *Nitrobacter*.

Nitrite was estimated, and nitrate detected, by a drop method with Griess-Ilosvay reagent [6]).

Results. A. With filtered glucose. The results of a typical experiment in which glucose sterilised by filtration was added to one of a pair of cultures, are given in Table I. This shows that nitrification was complete in both cultures, but that its onset was delayed in the glucose-treated culture; a delay of this kind was always found when filtered glucose was added before inoculation.

TABLE I

Filtered Glucose added before Inoculation (Expt. 4)

Days after Inoculation	Control		Glucose-treated	
	Nitrite (mg N/l)	Nitrate	Nitrite (mg N/l)	Nitrate
3	40		0	
4	75		0	
7	100		tr	
10	40		5	
12	1		8	
14	0	present	15	
17			50	
21			20	
24			0	present

To find out whether the glucose had damaged the nitrifying bacteria, subcultures were made from the glucose-treated cultures back on the usual medium without glucose; in every case these subcultures produced nitrite, and then nitrate, as quickly as the controls. More ammonium sulphate was added to a pair of cultures in which nitrification had been completed, and this second addition was nitrified at the same rate in the culture which had previously received glucose, as in the control culture.

So that it is evident that no permanent damage is done to the nitrifiers by filtered glucose, and the delay in nitrification cannot be due to its toxicity. A probable cause of the delay was discovered by making counts of the bacteria developing on nutrient agar in both cultures, in the first week after inoculation. Nutrient agar, which contains peptone, is so toxic to nitrifying bacteria that it is used to test the purity of nitrifying cultures [2]). Counts made on it therefore indicate the numbers of non-nitrifiers present. The results of a series

of such counts, given in Table II, show that glucose causes an enormous increase in the numbers of non-nitrifiers.

TABLE II

Counts on Nutrient Agar Plates			
Expt. No.	Days after Inoculation	Number of Bacteria (millions/ml)	
		Control	Filtered glucose
1	6	3.7	190
2	4	0.6	963
4	7	1.4	858
5	4	1.3	491
			Autoclaved glucose
6	7	5.3	740

Tests made on glucose-treated cultures at the time nitrite began to appear showed that no glucose was left in the medium. So it seems probable that the delay in nitrification was due to the swamping of the slow-growing nitrifiers by the rapid growth of the other species, and that when the glucose is used they die down, and ammonia released by autolysis is used by the nitrifiers.

Rather different results were obtained when glucose was added to an old culture, in which the nitrifying population had been built up by successive additions of ammonium sulphate and calcium carbonate[10]). Table III gives the results of an experiment in which a fourth addition of ammonium sulphate was made to a pair of cultures which had been incubated for three months, and at the same time filtered glucose was added to one culture. Nitrite was formed equally rapidly in both cultures, but there was a slight delay in the formation of nitrate in the glucose culture.

TABLE III

Filtered Glucose added to an Old Culture		
Days after addition *)	Nitrite (mg N/l)	
	Control	Glucose-treated
1	30	50
2	75	75
4	100	100
8	tr	75
15		5
23		0

*) Additions: 4 ml 0.66% $(NH_4)_2SO_4$ to both cultures.
2 ml 3.6% filtered glucose to glucose culture.

B. With Autoclaved Glucose. Table IV gives the results of an experiment in which the same concentration of glucose (0.02 M) was added to the medium and autoclaved with it. It will be seen that nitrification was completely stopped in the glucose culture; traces of nitrite appeared at first, but soon disappeared, and no more nitrite or nitrate was found, even after 32 days' incubation.

TABLE IV

Autoclaved Glucose in Medium before Inoculation				
Days after Inoculation	Control		Glucose-treated	
	Nitrite (mg N/l)	Nitrate	Nitrite (mg N/l)	Nitrate
2	40		tr	
3	75		tr	
4	100		0	
5	60		0	
7	0	present	0	absent
16			0	absent
32			0	tr

From the last line of Table II it will be seen that the autoclaved glucose produced a large increase in the number of non-nitrifying bacteria. But the failure of nitrification was not due simply to the growth of other species, as was shown by the results of subculturing. Subcultures from the cultures treated with autoclaved glucose back on to the usual glucose-free medium were not normal; either they failed to nitrify at all, or produced some nitrite after a long delay. So the nitrifiers are evidently poisoned by this comparatively low concentration of autoclaved glucose.

Discussion. The results reported in this paper show that glucose sterilised by filtration is not toxic to the nitrifiers, but that the same concentration autoclaved in the medium is toxic. The first work on this subject, by Winogradsky and Omelianskyi[11]) attracted much attention when it appeared, as it was thought so extraordinary that a good nutrient like glucose should be toxic to bacteria in such low concentrations; these authors found that 0.2% stopped oxidation by *Nitrosomonas*, and 0.3% by *Nitrobacter*. They did not say exactly how they sterilised their media, but from the context it was evidently by heat. Heubült[3]) also found that glucose autoclaved in the medium was toxic, but Meyerhof[7])

obtained the quite different result that 5% glucose had no effect on the respiration of *Nitrosomonas*; he used the Warburg apparatus, and presumably did not sterilise his reagents. Nelson [8]) compared two methods of sterilisation; if glucose was autoclaved in the medium 0.5% stopped oxidation by pure cultures of *N. monocella*, and 1% killed the bacteria, but glucose autoclaved separately and added to a growing culture was not toxic. Kingma Boltjes [5]) autoclaved glucose separately in distilled water, and found that 4% made no difference to the rate of ammonia oxidation. Recently Jensen [4]) has found that a *Nitrosomonas* from farmyard manure can tolerate 10% glucose sterilised by filtration, but ammonia oxidation is stopped by 1.5–2.5% autoclaved glucose. It therefore appears that the toxic effect of glucose on the nitrifiers depends on the method of sterilisation; that is, the toxic substance is not glucose itself, but a decomposition product formed by heating glucose in media. My results show that this toxic substance affects the nitrifiers in presence of other bacteria.

This is an interesting parallel to the case of the *Cytophagas* (Stanier [9]), which are poisoned by glucose autoclaved in the medium, but feed on it if sterilised by filtration; though the nitrifiers, which are autotrophic, do not use glucose, and added glucose can be recovered intact from pure cultures [8]).

Another point which arises from these results is that physiological work done with impure cultures needs very cautious interpretation. At first sight it might be thought that filtered glucose is toxic to nitrifiers, since it delays nitrification in mixed cultures; but subcultures are normal, which shows that the nitrifiers are not poisoned. The nutrient agar counts show that the effect of filtered glucose is exerted directly on the non-nitrifiers, and its effect on nitrification is indirect. If the non-nitrifying species cannot be ignored in cultures, how much more is this the case in soil? Here an apparent toxicity may be due to competition, or an apparent stimulation may be due to some other cause. Engel [1]) found that nitrification was increased by adding glucose to soil, but showed that this was due, not to any direct effect on the nitrifiers, but to the increased numbers of nitrogen-fixing bacteria, which provided more material for the nitrifiers to work on. Nitrification studies in soil have the advantage that the nitrifying bacteria are in a more or less natural environment; but the results obtained can be extremely confusing, if the very complex nature of the soil microflora is overlooked.

Summary

1. 0.02 M glucose sterilised by filtration delays nitrification in mixed cultures.

2. Filtered glucose is not toxic to nitrifying bacteria, so the delay is presumably caused by the rapid growth of non-nitrifying bacteria.

3. 0.02 M glucose autoclaved in the medium stops nitrification, and is toxic to the nitrifying bacteria.

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AMENDMENT TO GERRETSEN'S TECHNIQUE FOR THE PREPARATION OF PLATES USED FOR STUDYING PHOSPHATE-DISSOLVING MICRO-ORGANISMS

by H. W. JOHNSTON

Soil Bureau, Wellington, New Zealand

Gerretsen's paper [2]) on the influence of micro-organisms on the phosphorus uptake of plants gives a method for the preparation of plates which can be used to demonstrate the activity of phosphate-dissolving bacteria.

The instructions for the preparation of these plates read "... after sterilizing, the agar was cooled to 50°C and quickly mixed first with 0.5 cc 10 per cent K_2HPO_4 then 1.0 cc 10 per cent $CaCl_2$, a homogeneous precipitate of $CaHPO_4$ being formed". In repeating this technique, these instructions were thought to refer to a litre of medium and the resulting opacity due to precipitation of insoluble phosphate is negligible. The plates, when cool, do not resemble the plates shown in photographs accompanying Gerretsen's paper. To obtain plates of the required opacity the prescribed quantities of K_2HPO_4 and $CaCl_2$ should be added to each plate of medium in the order mentioned, mixed quickly and allowed to cool.

Using plates prepared in this way, attempts were made to isolate phosphate-dissolving bacteria by laying non-sterile roots on the sterilized medium, which was carrot agar, adjusted to pH 8.0 with caustic soda before sterilization. In every case the plates developed a fungal flora so readily that it was impossible to observe the action of rhizospheric bacteria.

Gerretsen did not mention any steps taken to control the pH of the carrot agar but in the reaction inferred, namely:

$$K_2HPO_4 + CaCl_2 = CaHPO_4 + 2KCl$$

it did not seem likely that any of the reactants involved would have much effect on the pH of the carrot agar, for KCl is neutral and $CaHPO_4$ is insoluble.

Nevertheless, the persistant appearance of fungi on plates which had been deliberately made alkaline, suggested that a shift in pH had occurred during precipitation of the phosphate. This was checked and it was found that after precipitation the pH had fallen to 6.0.

The solutions of K_2HPO_4 and $CaCl_2$ gave a pH of 9.0 and 7.0 respectively, and upon mixing 0.5 ml of 10 per cent K_2HPO_4 with 1.0 ml of 10 per cent $CaCl_2$, a pH of 5.4 resulted irrespective of whether the reaction took place in water or agar. In the next stage, 15 ml carrot agar was adjusted with caustic soda to give a pH range between 5.4 and 9.1. The pH after precipitation of phosphate is shown in Table I.

TABLE I

Reduction in pH in 15 ml carrot agar after precipitation of phosphate.				
pH before precipitation	5.4	6.8	8.6	9.1
pH after precipitation	5.3	6.2	6.4	6.8

This gave a clear indication that the equation assumed for the reaction did not hold and that from some source free acidity was produced. This acidity could not result from dissociation of dissolved $CaHPO_4$ because the solubility product of this compound is so low ($10^{-6.24}$)([3]), p. 232), and the dissociation constant $HPO_4^{=} \rightleftarrows H^+ + PO_4^{\equiv}$ is also very low (4.8×10^{-13}) ([4]), p. 1366), the amount of hydrogen ion contributed by $CaHPO_4$ in saturated solution cannot appreciably affect the pH of the water in which it is dissolved, — a fact which was demonstrated by experiment.

The fact that a mixture of 10 per cent K_2HPO_4 and 10 per cent $CaCl_2$ in the proportions of 1 : 2 by volume produces a system of pH 5.4 indicates that the precipitate cannot be entirely $CaHPO_4$, and in a reaction of this nature, where a cation has the capacity to form several insoluble compounds with slightly differing solubility products, it is likely that some of that compound with the lower solubility product will be formed. In the above reaction tricalcium phosphate can be formed thus:

$$3K_2HPO_4 + 3CaCl_2 = Ca_3(PO_4)_2 + H_3PO_4 + 6KCl$$

In view of the stoichiometrical properties of the reacting chemicals (1 : 4 of $K_2HPO_4.3H_2O$ and anhydrous $CaCl_2$) it is also possible that the reaction might proceed,

$$2K_2HPO_4 + 3CaCl_2 = Ca_3(PO_4)_2 + 4KCl + 2HCl$$

Therefore, acidity produced during the reaction between K_2HPO_4 and $CaCl_2$ may be taken as an indication that a *mixture* of calcium phosphates is formed, as indeed is to be expected ([1]), p. 733).

Gerretsen's results therefore refer to solubilization of a *mixture* of insoluble phosphates and no distinction can be drawn between the capacity of the bacteria for dissolving any particular form of phosphate in the mixture. It does not necessarily follow that an organism which renders $CaHPO_4$ soluble can, with equal facility, be effective on $Ca_3(PO_4)_2$.

A slight amendment to Gerretsen's method allows this distinction to be made. If, instead of precipitating phosphate in the medium, a very fine water suspension of $CaHPO_4.2H_2O$ or $Ca_3(PO_4)_2$ is added to a plate of medium and mixed thoroughly before the agar sets, accurate control over the type of phosphate present is assured.

The suspension is easily prepared by wet grinding 5 g $CaHPO_4$ or $Ca_3(PO_4)_2$ in a ball mill for several hours. The extremely finely divided phosphate is washed out and made up to 500 ml with water. 1 ml of this stock suspension in 15 ml carrot agar gives a medium of suitable opacity for microbiological work.

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